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(54) Title: NOVEL PYRUVATE COMPOUNDS AND METHODS FOR USE THEREOF (57) Abstract The invention comprises a novel pyruvate compound for the treatment or prevention of reperfusion injury following ischemia, diabetic effects, cholesterol levels, injured organs, ethanol intoxication, or as a foodstuff. The novel pyruvate compound is particularly a pyruvate thiolester, a glycerol-pyruvate ester or a dihydroxyacetone-pyruvate ester.		

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NOVEL PYRUVATE COMPOUNDS AND METHODS
FOR USE THEREOF

This application is a continuation-in-part of pending U.S. Serial No. 617,255, filed March 18, 1996.

Background of the Invention

This invention relates to several new pyruvate
5 compounds and methods of treating (i) ischemia in
mammalian hearts, lungs, veins, arteries and other
organs or tissues, (ii) accelerating ethanol
oxidation/preventing acute toxic effects of ethanol on
the liver, and (iii) other recognized uses of pyruvates
10 including, but not limited to, treating of secondary
effects of diabetes, lowering of cholesterol and lipid
levels, as a nutrition source which can provide as much
as 100% of caloric requirements and to treat injured
organs requiring a readily accessible energy source.

15

Description of the Art

Ischemia is defined herein as the interruption of
oxygen supply, via the blood, to an organ or to part of
an organ. Examples of ischemic events include (i)
myocardial, cerebral, or intestinal infarction following
20 obstruction of a branch of a coronary, cerebral, or
mesenteric artery, and (ii) removal and storage of an
organ prior to transplantation. In the case of

myocardial infarction, prompt restoration of blood flow to the ischemic myocardium, i.e. coronary reperfusion, is a key component of the treatment. This is because mortality is directly related to infarct size (tissue
5 necrosed) which is related to the severity and duration of the ischemic event.

Notwithstanding the need to supply an organ cut-off from a normal blood supply with oxygen, it has been found that reperfusion injury may occur upon restoration
10 of blood flow. This results from the production of reactive oxygen species (ROS), namely, hydrogen peroxide, hydroxyl radicals and superoxide radicals which are formed from both extracellular and intracellular sources. ROS are highly reactive species
15 that, under normal conditions, are scavenged by endogenous defense mechanisms. However, under conditions of post-ischemic oxidative stress, ROS interact with a variety of cellular components, causing peroxidation of lipids, denaturation of proteins, and
20 interstitial matrix damage, resulting in increase of membrane permeability and release of tissue enzymes.

In an attempt to minimize these undesirable side effects of perfusion, researchers Simpson, et al., (Free Radical Scavengers and Myocardial Ischemia, Federation
25 Proceedings, Volume 46, No. 7 May 15, 1987) suggest the use of an inhibitor of ROS production to protect the reperfused myocardium. The Simpson, et al. disclosure is particularly directed to the use of agents and

inhibitors (ex. allopurinol) that reduce ROS levels. In a similar context, Brunet, et al., (Effects of Acetylcysteine, *Free Radical Biology and Medicine*, Volume XX, No. X 1995) suggest the use of acetylcysteine to reperfuse hearts. In particular, the article concludes that acetylcysteine treatment decreases the production of ROS in reperfused rat hearts.

In a further effort directed to protecting reperfused heart tissue, United States Patent 5,075,210, herein incorporated by reference, discloses a process for reperfusing a heart for transplantation. The patent discloses a cardioplegic solution containing sodium chloride, potassium chloride, calcium chloride, sodium bicarbonate, sodium EDTA, magnesium chloride, sodium pyruvate and a protein.

United States Patent 5,294,641, herein incorporated by reference, is directed to the use of pyruvate to prevent the adverse effects of ischemia. The pyruvate is administered prior to a surgical procedure to increase a patient's cardiac output and heart stroke volume. The pyruvate is administered as a calcium or sodium salt. The pyruvate can alternatively be an ester of pyruvic acid such as ethylamino pyruvate. Similarly, United States Patent 5,508,308, herein incorporated by reference, discloses the use of pyruvyl glycine to treat reperfusion injury following myocardial infarction.

United States Patent 4,988,515 and 5,705,210, herein incorporated by reference, use pyruvate salts in

cardioplegic solutions and in preservation solutions for the heart before transplantation. United States Patent 4,970,143, herein incorporated by reference, discloses the use of acetoacetate for preserving living tissue, including addition of the pyruvate to the preservation solution.

United States Patent 5,100,677 herein incorporated by reference, discloses the composition of various parenteral solutions. Of interest is a recommendation to include pyruvate anions (apparently from metal salts) in intravenous solutions. In United States Patent 5,183,674, herein incorporated by reference, pyruvate compounds are used as foodstuff. United States Patent 5,134,162 herein incorporated by reference, focuses on the use of pyruvate to lower cholesterol and lipid levels in animals. United States Patent 5,047,427, deals with the use of pyruvate for improving the condition of diabetics, while United States Patent 5,256,697 suggests the use of pyruvyl-aminoacid compounds, each of which is herein incorporated by reference.

In addition, United States Patent 5,283,260, herein incorporated by reference, is directed to treatment of diabetes with a physiologically acceptable form of pyruvate. The patent discloses a pyruvate compound in the form of a covalently linked pyruvyl-amino acid. By utilizing this type of a pyruvate delivery system, the negative effect of pyruvate salt is avoided. However,

administration of large amounts of pyruvate-amino acid may result in nitrogen overload which could harm patients with liver and/or kidney pathology.

Notwithstanding the acceptance of pyruvate as an
5 effective component of a reperfusion solution or other varied applications, pyruvic acid is a strong and unstable acid which cannot be infused as such. Furthermore, it has been recognized that traditional pharmacological pyruvate compounds, such as salts of
10 pyruvic acid, are not particularly physiologically suitable. For example, these compounds lead to the accumulation of large concentrations of ions (ex. calcium or sodium) in the patient's body fluids. Similarly, amino acid compounds containing pyruvate can
15 lead to excessive nitrogen loads. It has also been proposed to infuse pyruvylglycine, the amide function of which is presumably hydrolyzed in plasma and/or tissues, thus liberating pyruvate. However, at the high rates of pyruvylglycine infusion required to achieve 1 mM
20 pyruvate in plasma, the glycine load may be harmful to patients suffering from hepatic or renal pathologies. Also, flooding plasma with glycine may interfere with the transport of some amino acids across the blood-brain barrier. Accordingly, while potentially suitable to
25 organ preservation, these pyruvate compounds are less suited to treating an organ in vivo, and it is recognized that a need exists to provide a pyruvate

delivery compound which is more physiologically acceptable.

Therefore, it is desirable in this field to have an alternate physiologically compatible therapeutic
5 pyruvate compound. In this regard, the novel pyruvate compounds of this invention permit the use of pyruvate to treat ischemic events, ethanol poisoning, acetaminophen poisoning and other recognized pyruvate effective treatments because sufficiently high loads of
10 pyruvate can be administered without a toxic constituent.

Summary of the Invention

Accordingly, it is a primary object of this invention to provide a new and improved pyruvate
15 compound(s).

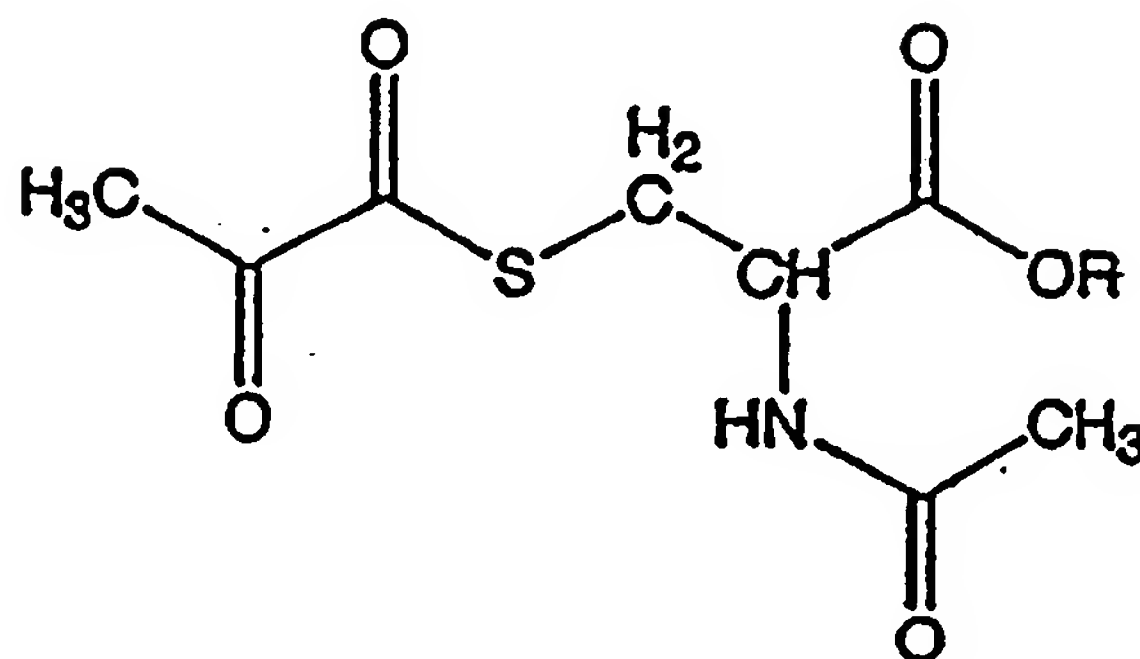
It is an advantage of this invention to provide a new and improved method for organ reperfusion. It is still a further advantage of this invention to provide a new and improved method for treating ethanol
20 intoxication. An additional advantage of this invention is to provide a pyruvate compound which can provide nutritional benefits.

To achieve the foregoing objects and in accordance with the purpose of the invention, as embodied and
25 broadly described herein, one novel pyruvate compound of this invention comprises a pyruvate thiolester. Preferably, the thiol is cysteine or homocysteine. In

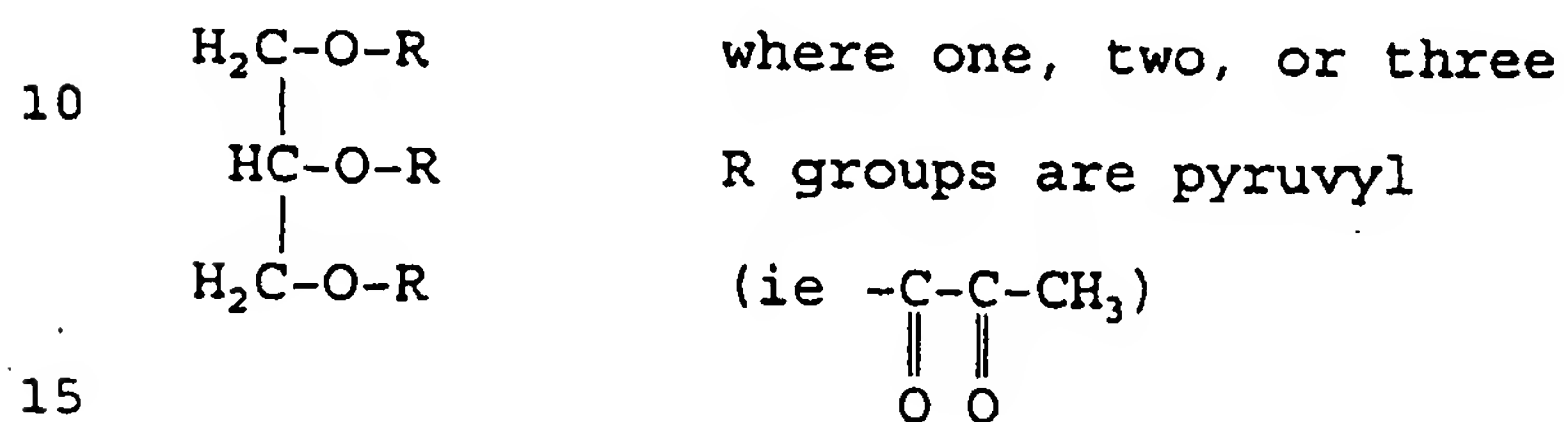
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a particularly preferred form, the compound is a N-acetyl ethyl ester of the cysteine or homocysteine amino acid.

One preferred compound is:

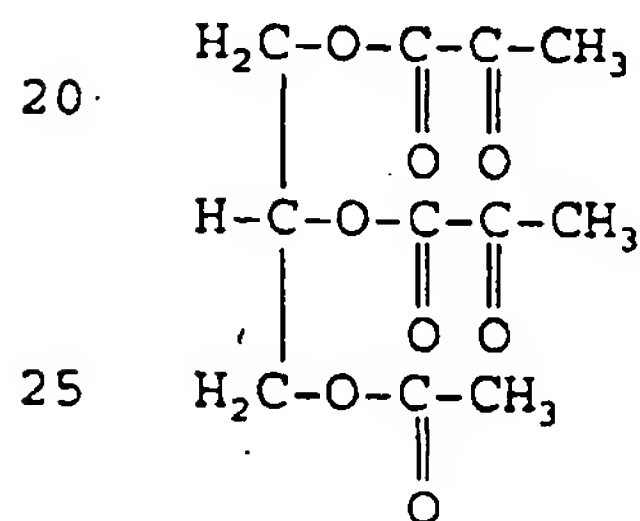


5 A further novel compound of the present invention is a glycerol-pyruvate ester. A particularly preferred form of a glycerol-pyruvate ester will be of the formula:



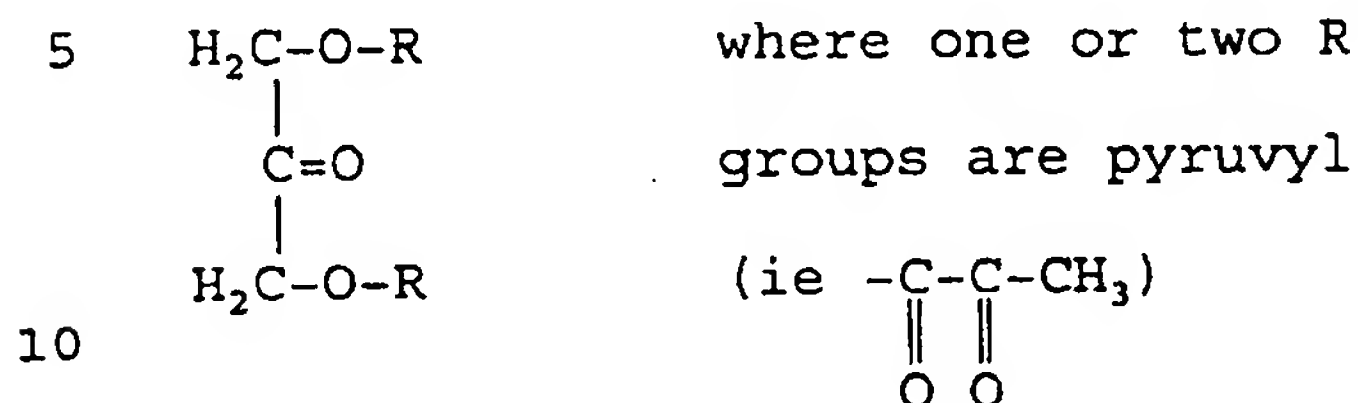
and one or two R may be a short-chain acyl such as acetyl or propionyl.

and more preferably



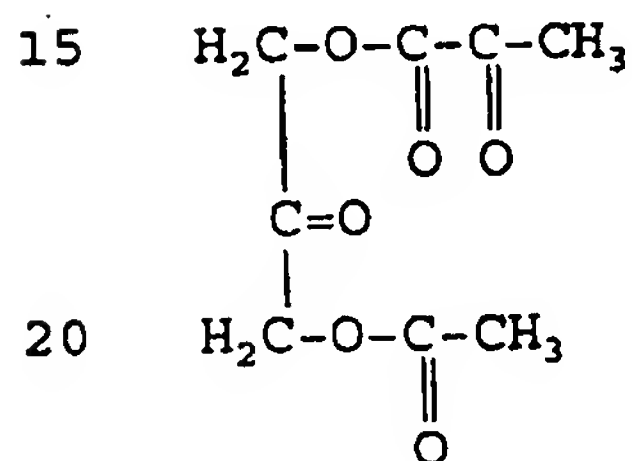
Another novel compound of the present invention is a dihydroxyacetone-pyruvate ester.

A particularly preferred form of this compound is of the formula:



and one R may be a short-chain acyl such as acetyl or propionyl.

and more preferably



The invention is also directed to use of the novel pyruvate compounds in reperfusion of tissue and organs both in vivo and in storage. Accordingly, the invention includes a method for the preservation of tissue deprived of oxygen through events including, but not limited to, coronary infarction, stroke, mesenteric infarction, organ transplant (during preservation and intravenously after grafting of the organ) including amputated limbs. The compound is also believed well suited to treatment of acetaminophen poisoning of the

liver which depletes liver glutathione stores leading to acute hepatic necrosis.

This invention is also directed to the use of the novel pyruvate compounds to assist a patient's body in ethanol oxidation. In fact, the novel pyruvate compounds of this invention are suited to use as nutritional supplements, preventing body fat deposition, lowering high blood cholesterol levels, and treatment for secondary diabetes effects.

It is believed that pyruvate acts as a NADH trap and a trap for ROS generated upon reperfusion. In addition, the thiol group from cysteine, for example is believed to scavenge ROS. Similarly, the carbonyl group of dihydroxyacetone acts as a NADH trap. Accordingly, the subject novel compounds provide stable, and physiological compounds with the beneficial result of delivering pyruvate and an additional NADH and ROS trapping moiety.

Brief Description of the Drawings

The invention consists of the novel parts, construction and arrangements, combinations and improvements shown and described. The accompanying drawings, which are incorporated in and constitute a part of the specification illustrate one embodiment of the invention and together with the description explain the principals of the invention.

Of the drawings:

FIGURES 1 and 2 are graphical representations of the results of the experiments on ischemia of isolated rabbit hearts set forth hereinbelow;

FIGURES 3 - 7 show the results of experiments on ethanol oxidation in livers;

FIGURES 8 and 9 show the results of experiments directed to pyruvate concentrations in arterial blood; and

FIGURE 10 shows the results of experiments on the effect of DPAG on ethanol uptake.

Detailed Description of the Preferred Embodiments

For the purposes of this disclosure, the following abbreviations are used:

ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; DCA, dichloroacetate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; DPAG, dipyruvyl-acetyl-glycerol; FAEE, fatty acid ethyl esters; GC, gas chromatography; GCMS, gas chromatography-mass spectrometry; LAD, left anterior descending coronary artery; MPE, molar percent enrichment; MS, mass spectrometry; NAC, N-acetylcysteine; NEFA, non-esterified fatty acids; PADA, pyruvyl-acetyl-dihydroxyacetone; PDH, pyruvate dehydrogenase; PNACE, pyruvate N-acetylcysteine ethyl ester; ROS, reactive oxygen species.

Reperfusion

As described above, timely coronary reperfusion as treatment for acute myocardial infarction reduces myocardial infarct size and improves survival rates. However, there is concern that reperfusion may cause further injury to the myocardium, called "reperfusion injury". More particularly, experimental studies have demonstrated that myocardium reperfused after reversible ischemia exhibits prolonged depression or "myocardial stunning". There is evidence that reperfusion of ischemic myocardium results in the generation of ROS and that a burst of ROS production at the time of reperfusion causes myocardial damage. Accordingly, attempts have been made to provide pyruvate compounds which trap and/or prevent the formation of ROS.

One form of the present invention is a novel compound including a pyruvate moiety which also traps reducing equivalents (NADH) and ROS, and a thiol moiety which traps ROS. Accordingly, the present compound provides dual functionality in an effective and highly efficient manner and in a physiologically soluble molecule. In addition, the compounds are degraded to physiological and safe metabolites (pyruvate, cysteine or homocysteine). Lastly, the present inventive compound is equally applicable to use in the preservation of organs removed for transplantation.

In summary, the novel compounds are redox chimeras whose molecules contain a trap for reducing equivalents (NADH) and a trap for ROS.

The inventive compounds demonstrate the following
5 characteristics;

- (i) water solubility;
- (ii) no ionic charge, to facilitate diffusion through cell membranes and to avoid the need to administer a counter-ion, such as Na⁺;
- 10 (iii) metabolizable to physiological compounds; and
- (iv) stability in solution.

PNACE

One preferred group of the inventive compounds is a thiolester of pyruvate and a sulfur amino acid, for
15 example cysteine or homocysteine. Preferably, any ionizable functions on the amino acid molecule are blocked by easily removable radicals, such as ethyl and N-acetyl groups. The most preferred compound is formed of pyruvate and N-acetylcysteine ethyl ester.

20 The invention will now be described with reference to the following examples, intended to describe, but not limit the invention.

Recovery of isolated rabbit hearts following 25 min of warm ischemia

25 Hearts were removed from anesthetized New Zealand rabbits (2.5 - 3.0 kg) and perfused in the working mode at 37° with non-recirculating Krebs-Ringer bicarbonate

(KRB) buffer containing 5 mM glucose and 5 units/l of insulin, and equilibrated with a gas mixture containing 95% O₂ + 5% CO₂. In the working mode, which simulates physiological conditions, hearts pump the buffer against
5 a 85 cm hydrostatic pressure in the aorta. The mechanical performance of the hearts was assessed by monitoring heart rate, cardiac output, coronary flow, left ventricular pressure, and dp/dt_{max} . The latter parameter reflects the capacity of the heart to increase
10 hydrostatic pressure in the left ventricle.

Following 30 minutes of equilibration, the hearts were made ischemic for 25 minutes by clamping the aortic and left atrial canulas. Then, the clamps were removed to allow reperfusion with oxygenated KRB buffer
15 containing either no additive (n=7, control group), or 20 μ M pyruvate-N-acetyl-cysteine ethyl ester (PNACE) (n=7). PNACE was infused via a syringe pump into the inflowing perfusate. In the syringe, PNACE was dissolved in 0.1 mM HCl to prevent hydrolysis of the
20 thiolester.

None of the control group hearts recovered any function. In contrast, hearts reperfused with buffer containing 20 μ M PNACE recovered 75 to 95% of their mechanical function after reperfusion was instituted
25 (see Figs. 1 and 2). Functional recovery lasted throughout the 30 minute reperfusion experiment.

Recovery of isolated rabbit hearts following massive catecholamine injury

Rabbit hearts were perfused in the working mode as in the above example. However, after the 30 minutes of equilibration, 50 μ M isoproterenol was added to the inflowing perfusate for 10 min. Isoproterenol is a catecholamine, which, at the dose administered, induces a marked increase in heart rate and cardiac output. After 10 minutes, the mechanical performance of the hearts decreased markedly to the point where cardiac output was almost zero. Then, isoproterenol infusion was stopped, and perfusion was continued for 30 minutes with oxygenated KRB buffer containing either no additive (n=7, control group), or 20 μ M PNACE (n=7). The hearts perfused with plain buffer did not show recovery of cardiac function. In contrast, hearts perfused with buffer containing 20 μ M PNACE recovered 75 to 95% of their mechanical function.

The data of these experiments proved substantially similar to that shown in FIGURES 1 and 2.

Improved function of preserved rat livers

Livers from overnight-fasted rats were surgically removed and flushed at 37°C with non-recirculating KRB buffer containing 5 mM glucose and equilibrated with a gas mixture containing 95% O₂ + 5% CO₂. The first group of livers (n=7, control group) was not preserved, but was perfused at 37°C for 45 minutes. The second group of livers (n=8, preserved group) was flushed with ice-

cold University of Wisconsin (UW) preservation solution and stored for 24 hours in ice-cold UW solution. Next, the livers were reperfused at 37°C with non-recirculating KRB buffer containing 5 mM glucose. The
5 third group of livers (n=8, preserved + PNACE group) was treated as the second group except that 20 μ M PNACE was added to the UW preservation solution and to the reperfusion KRB buffer. During the last 45 min of (re)perfusion, the function of the three groups of
10 livers was assessed by (i) the release of three cellular enzymes, i.e. lactate dehydrogenase, aspartate aminotransferase, and alanine aminotransferase, (ii) oxygen consumption, and (iii) the production of ketone bodies, ie β -hydroxybutyrate + acetoacetate, after
15 addition of 1 mM octanoate to the perfusate.

The data depicted in the Table, show that, in preserved reperfused rat livers, PNACE (i) markedly decreases the initial release of tissue enzymes, (ii) restores partially the capacity of the liver to oxidize
20 fatty acids to ketone bodies, and (iii) restores oxygen consumption to the level of non-preserved livers.

Effect of PNACE on metabolic integrity of preserved rat livers upon reperfusion

All data from group III are statistically different from the corresponding data of group II.

5	Parameter measured	Group I Control non-preserved (7)	Group II Control preserved (8)	Group III Preserved + PNACE (8)
	Release of lactate dehydrogenase (U/L.g) 10-14 min.	1.9	21.8	4.5
10	Release of aspartate amino-transferase (U/L.g) 25-30 min.	0.28	3.4	0.73
15	Release of alanine aminotransferase (U/L.g) 25-30 min.	0.21	3.7	0.33
	Ketone body production (μ mol/min.g) 25-30 min.	3.1	0.9	1.4
20	Oxygen consumption (μ mol/min.g) 25-30 min.	2.1	1.3	2.4

Synthesis of PNACE

As understood in the art, pyruvate has proven to be a relatively unstable compound with very limited mechanism for satisfactory delivery to subjects. However, the present inventive compound has proven to be readily manufacturable and very effective in the prevention of organ damage associated with reperfusion injury. The compound has been prepared in pure form and in gram amounts. Its formula has been confirmed by elemental analysis and gas chromatography-mass spectrometry. The compound is stable in slightly acidic solutions (pH 4-5). At pH 7.4, it is slowly hydrolyzed to pyruvate and N-acetylcysteine ethyl ester. The compound has also been synthesized labeled with three deuterium ^2H atoms on the N-acetyl moiety. This

deuterated compound is used as an internal standard for the assay of the compound by isotope dilution gas chromatography-mass spectrometry.

In a three-neck flask of 500 ml, freshly distilled
5 pyruvic acid (9.06 g., 0.102 mol) and N-hydroxy-succinimide (11.82 g., 0.102 mol) in dry tetrahydrofuran (THF) (180 ml) was stirred under nitrogen and was cooled in a ice bath. Dicyclohexylcarbodiimide (21.2 g., 0.102 mol) dissolved
10 in dry THF (150 ml) was added slowly to the stirred cooled mixture over approximately 1 hr. Then, the reaction mixture was stirred at room temperature for 2.5 hr, followed by slow addition of N-acetyl-L-cysteine ethyl ester (6.81 g., 0.033 mol) dissolved in 20 ml dry
15 THF over approximately 1 hr. The reaction mixture was stirred overnight at room temperature under a nitrogen atmosphere.

After evaporating the THF, the residue was suspended in ethyl acetate (750 ml) and was kept for 4-6
20 hr at 0°C. Dicyclohexyl urea (DCU) was then filtered and discarded; the ethyl acetate solution was washed three times with water (3x100 ml). It was then dried over anhydrous sodium sulfate and concentrated under vacuum.

25 The product (17-18 g.) was further purified by using column chromatography. A column of 5 cm. diameter was filled with silica gel (180-200 g., 60 Angstrom flash chromatography from Aldrich). The product was

dissolved first in a minimum quantity of ethyl acetate:hexane (60:40) and was loaded on the column. The column was developed under gravity (rather than flash chromatography) with ethyl acetate:hexane (60:40).
5 Fifty ml fractions were collected and monitored by TLC using either iodine or UV light. The fractions containing the product were combined and solvents were removed under reduced pressure. The residue was dissolved in chloroform (300 ml), first washed with 5%
10 HCl (2x30ml) and then saturated NaCl (3x60 ml). The organic layer was dried over anhydrous sodium sulfate, filtered, and the solvent evaporated. The residue was dissolved in a minimum quantity of chloroform, and petroleum ether was added until the solution became
15 turbid. The suspension was kept overnight in the refrigerator and then filtered to get the pure crystallized product. The compound was dried under vacuum over P_2O_5 to a yield of 6.5 g. (75%, based on the N-acetyl-L-cysteine), m.p. 76-77°C.

20 Alternative Synthesis of PNACE

To a 250 ml three neck flask fitted with a thermometer, a magnetic stirrer, a 50-ml pressure-compensated addition funnel, and a Friedrich's condenser under nitrogen, was added 10g (52.3 mmoles) of N-acetyl-L-cysteine ethyl ester, 8.0 ml of dry pyridine and 60 ml
25 of dry benzene.

Pyruvoyl chloride (0.104 mole, 2 eq) was added dropwise over a period of 0.5 hr. while maintaining a temperature of 5°C to 10°C. Then, the reaction mixture was allowed to warm to 25°C and stirred for 2 hours at this temperature. The benzene solvent was then evaporated under vacuum. The crude product was purified as above to yield 11.15 g of the desired compound (82%).

Synthesis of Deuterated PNACE

Pyruvate-N-[²H₃]acetyl-L-cysteine ethyl ester

10 Synthesized wherein the above procedure was followed using N-[²H₃] acetyl-L-cysteine ethyl ester to form (d₃-PNACE). The latter was prepared by reacting L-cysteine ethyl ester with [²H₆]acetic anhydride.

15 Set forth hereinbelow are certain analytical characteristics of the composition of the invention provided to facilitate identification thereof, but not intended to limit the scope.

Characteristics of Compounds

I. Pyruvate-N-acetyl-L-cysteine ethyl ester : PNACE
20 (unlabeled)

mp : 65°C

Rf (ethyl acetate/petroleum ether: 3/2) : 0.52

IR (Nicolet 300, CCl₄) (cm⁻¹) :

3435 (ν N-H)

25 3000 (ν C-H)

- 20 -

1747 (ν CO-O) ester1731 (ν CO-S) thioester1687 (ν CO-CO, CO-N) ketoester, amide

1497, 1378.3, 1210.1

5	NMR ^1H , 300MHz (Varian, CDCl_3 , TMS) (ppm):	NMR ^{13}C , 100.12 MHz (Bruker, CDCl_3 , TMS) (ppm):
	1.33 (t, $^3J=7.13$, 3H, OCH_2CH_3)	190.6; 188.08 keto, ketoester
	2.10 (s, 3H, COCH_3)	168.6, 168.08 ester, amide
	2.50 (s, 3H, CH_3COCO)	60.1 (OCH_2)
10	3.45 (dd, $^3J=4.10$ Hz, $^3J=8.95$ Hz, 2H, $\text{CH}_2\text{-S}$)	49.5 (CH_2S)
	4.23 (dd, $^3J=7.13$ Hz, 2H, CH_2CH_3)	28.4 (CHNH)
	4.83 (m, 1H, CH)	21.9 (CH_3COCO)
	6.50 (sl, 1H, NH)	20.8 (CH_3CO)
		12.1 (CH_3CH_2)
15	Mass spectrum, electron ionization (m/z):	Mass spectrum, ammonia chemical ionization (m/z):
	190 (M - CH_3COCO , 33); 118(26);	279 (M+18, 100); 262(M+1, 93); 209(49); 192(60)
	102(56); 76(33), 60(90), 43 (CH_3CO^+ , 100)	175(18), 158(26)

II. Pyruvate-N- $[\text{D}_2]$ acetyl-L-cysteine ethyl ester : d_3 -PNACE (deuterated)

20	NMR ^1H , 300MHz (Varian, CDCl_3 , TMS) (ppm) :	NMR ^{13}C , 100 MHz (Bruker, CDCl_3 , TMS) (ppm):
	1.34 (t, $^3J=7.13$, 3H, OCH_2CH_3)	190.5; 187.08 keto, ketoester
	2.50 (s, 3H, CH_3COCO)	168.5, 168.10 ester, amide
	3.42 (dd, $^3J=4.10$ Hz, $^3J=8.95$ Hz, 2H, $\text{CH}_2\text{-S}$)	60.1 (OCH_2)
	4.25 (dd, $^3J=7.13$ Hz, 2H, CH_2CH_3)	49.1 (CH_2S)
25	4.90 (m, 1H, CH)	28.1 (CHNH)
	6.50 (sl, 1H, NH)	20.8 (CH_3COCO)
		19.9 (CD_3CO)
		12.0 (CH_3CH_2)
30	Mass spectrum, electron ionization (m/z):	Mass spectrum, ammonia chemical ionization (m/z):
	193 (M- CH_3COCO , 17); 121(4); 103(29);	282(M+18, 42); 265(M+1, 47);
	77(12); 63(26); 43 (CH_3CO , 100)	212(23), 195(37); 178(53); 161(100);
		106(23); 89(15)

Ethanol Metabolism

35 The following is believed to represent aspects of the human system for ethanol oxidation, but is supplied only as a representation of the theory, and is not intended to limit the invention in any way.

40 Ethanol is oxidized to acetate in the liver and the stomach by two reactions catalyzed by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)

which use NAD^+ as coenzyme. The bulk of ADH activity is in the liver. ALDH activity appears to occur in most tissues.

The ADH reaction is reversible; the mid-potential of the ethanol/acetaldehyde couple is -230 mV, which is very close to that of the lactate/pyruvate couple (-225 mV). In the absence of ALDH, the plasma ethanol/acetaldehyde ratio would be similar to the lactate/pyruvate ratio (about 10). However, the equilibrium of ADH is displaced by ALDH, the equilibrium of which is far toward acetate because of the very negative mid-potential of the acetaldehyde/acetate couple (-265 mV). As a result, the ethanol/acetaldehyde ratio is very high (>1000) and the acetaldehyde concentration difficult to measure (in the low μM range) unless ALDH is inhibited by disulfiram. In this invention, strategies for accelerating ethanol oxidation target the ADH pathway, but may have an impact on some of the toxic effects derived from non-ADH pathways.

Ingestion of alcoholic beverages leads to ethanol concentrations in body fluids that are much higher than the K_m of liver ADH for ethanol. For example, in many US states, the legal limit of blood ethanol concentration compatible with driving a car, is 0.75 g/l or 17 mM. Drunkenness occurs at concentrations above 30 mM, and alcoholic coma at variable concentrations above 40 mM.

Reducing equivalents generated in the cytosol by ethanol oxidation are transferred to the mitochondria via the malate/aspartate and the citrate/malate shuttles. In mitochondria, reducing equivalents are oxidized in the respiratory chain. Ethanol oxidation can be seen as a sequence of three processes catalyzed by ADH, the reducing equivalent shuttles and the respiratory chain. It is possible to set up in vitro conditions where ethanol oxidation is limited either by ADH, the shuttles or the respiratory chain. However, in the intact in vivo liver, ethanol oxidation appears limited by the capacity of the respiratory chain to oxidize reducing equivalents, which itself is set by the ATP turnover. Under extreme conditions, most of the liver O_2 uptake is used to oxidize the reducing equivalents derived from ethanol.

Since the rate of ethanol oxidation is limited by the ATP turnover, an increase in this turnover raises the capacity to oxidize ethanol. In chronic ethanol ingestion (before liver decompensation), hyperthyroidism and chronic exposure to cold, the liver ATP turnover and the capacity of ethanol oxidation increase. In fact, a single gavage of ethanol induces (i) an increase in the rate of O_2 uptake by the rat liver perfused in the absence of ethanol, and (ii) an increase in the in vivo capacity of rats and humans to oxidize a second dose of ethanol.

In isolated livers, one can accelerate ethanol oxidation by imposing a drain on ATP with ureogenic substrates (NH_4Cl + ornithine + asparagine for example), gluconeogenic substrates or uncoupler of the respiratory
5 chain. In dogs, an intragastric gavage of NH_4HCO_3 increases the rate of ethanol oxidation. However, the toxicity of uncouplers precludes their use *in vivo*.

The liver of an adult human accounts for only 2% of body weight. However, the liver receives 100% of water-
10 soluble nutrients absorbed from the gut. In addition, it must handle part of the lipid material (i) absorbed from the gut via the lymphatic system and (ii) released by adipose tissue lipolysis. Only a small fraction of nutrients' energy is used in the liver. Most of this
15 energy is exported as substrate molecules to peripheral tissues.

The O_2 uptake of the 1.5 kg liver of a 75 kg subject is about 3 mol/day. Thus, the maximum ATP production of the liver is 18 mol/day. If all this O_2 uptake were used
20 to oxidize ethanol to CO_2 , only 1.1 mol of ethanol (51 g) could be oxidized per day. This would leave no room for (i) the obligatory hepatic ATP production from protein catabolism, (ii) ATP production from carbohydrate and fat catabolism. The liver manages this energy plethora
25 by exporting most of the potential energy of ethanol as acetate, thus decreasing by 80% the hepatic ATP production from ethanol. Acetate is well used in peripheral tissues.

While most ethanol can be exported from the liver, there is no large-scale mechanism for exporting reducing equivalents from the liver. One obvious export mechanism would (i) trap reducing equivalents in the conversion of pyruvate to lactate, and (ii) export
5 lactate to peripheral tissues. However, plasma pyruvate concentration is very low (0.05-0.1 mM). Pyruvate could be generated from glucose and amino acids, but these processes would further increase the liver's ATP burden.
10 For these reasons the exogenous pyruvate compounds of the present invention are particularly suited to assist the body with ethanol oxidation.

The unregulated production of reducing equivalents in the liver increases the cytosolic and mitochondrial
15 $[NADH]/[NAD^+]$ ratios. This redox shift inhibits gluconeogenesis from proteins and the recycling of glucose in the Cori cycle by displacing the equilibrium of lactate, malate and α -glycero-P dehydrogenases. If the hepatic glycogen reserves are exhausted (for example
20 after more than 12 hr of fasting), inhibition of gluconeogenesis can induce alcoholic hypoglycemia which can lead to hypoglycemic coma, brain damage and death. In addition, the redox shift inhibits the citric acid cycle at α -ketoglutarate dehydrogenase, lowering CO_2
25 production and the respiratory quotient sometimes to almost zero. Then, all the liver O_2 uptake is used to oxidize the reducing equivalents derived from ethanol.

Other processes are involved in the hepatic toxicity of ethanol: binding of acetaldehyde to proteins, damage to proteins by free radicals (superoxide, hydroxyl) probably generated by cytochrome P450 IIE1, which is induced by chronic alcohol ingestion. There is evidence that ethanol generates pro-oxidant reactive species in both the liver and the central nervous system. This leads to depletion of glutathione and to the covalent binding of hydroxyethyl radicals to liver microsomal proteins. Free radical damage and malnutrition are most likely implicated in the cirrhotic process.

Fatty acid ethyl esters (FAEE) have been identified in liver and in organs where oxidative metabolism of ethanol is minimal or absent, but which are commonly damaged by ethanol abuse: brain, pancreas, myocardium, and in cells cultured in the presence of ethanol. FAEE are formed by a synthase and an acyl-CoA:ethanol acyltransferase activity present in microsomes and cytosol of these organs. FAEE synthase activity is also present in white blood cells. After ethanol ingestion, FAEE are found in LDLs. Serum FAEE concentrations, assayed by GCMS, correlate with ethanol concentration. The half-life of plasma FAEE is ~1 min; they undergo hydrolysis in plasma and uptake by organs. FAEE bind to myocardial mitochondria in vitro and in vivo. The mitochondria hydrolyze FAEE to fatty acids, which are uncouplers of oxidative phosphorylation. This may

account for the impaired mitochondrial function and inefficient energy production associated with the toxic effects of ethanol on the heart. Also, the accumulation of FAEE in embryos of rats given ethanol has been linked
5 to the fetal-alcohol syndrome.

Amethystic agents could be used to treat alcoholic coma. Ethanol concentrations are typically in the 50 to 70 mM range. This condition is life threatening because it can mask alcoholic hypoglycemia leading to brain
10 damage and possibly death. In addition, anesthesia of the respiratory center by ethanol depresses respiration and coughing, leading to respiratory acidosis and pulmonary infection. Sometimes, respiratory depression leads to respiratory arrest. Also, general alcoholic
15 anesthesia induces hypothermia which itself can be lethal. Patients in alcoholic coma brought to the Emergency Room could be infused with amethystic agents until they regain some consciousness and show no depression of respiration. Similarly, if safe oral
20 amethystic agents were available, their ingestion between alcoholic drinks, or sometimes before driving an automobile, could dramatically decrease the rate of accidents or violence while under the influence.

Ethanol oxidation in liver is limited by the
25 capacity of the respiratory chain to oxidize reducing equivalents derived from the conversion of ethanol to acetate. The administration of pyruvate will accelerate ethanol oxidation by exporting the reducing equivalents

in the form of lactate. This export will shift the burden of disposing of reducing equivalents from the liver (2% of body weight) to the bulk of peripheral tissues. Lactate is well used by peripheral tissues, particularly by muscle and kidney. In these tissues, lactate oxidation to CO_2 will probably result in an underutilization of fatty acids, glucose and glycogen. The notion of Cori cycle, in which lactate derived from glycolysis in erythrocytes is recycled to glucose in the liver, does not exclude oxidation of a large fraction of this lactate in peripheral tissues.

Pyruvic acid is a strong, unstable ketoacid which cannot be administered orally or parenterally. Sodium pyruvate is stable in dry form and could be dissolved as an isotonic solution just before use. However, the sodium and water loads would kill the patient. The effects of the sodium load is compounded by the difference between the volumes of distribution of sodium (20% of body wt) and of ethanol (67% of body wt). Consider a numerical example: suppose we want to decrease the ethanol concentration by 20 mM in a 75 kg patient. The ethanol pool must decrease by $20 \times 75 \times 0.67 = 1,000$ mmol. Since the oxidation of 1 mol ethanol generates 2 NADH, we need to supply $2 \times 1,000 = 2,000$ mmol of sodium pyruvate as $2,000/150 = 13.3$ liters of isotonic solution (150 mM). Such volume cannot be administered safely. In addition, the 2,000 meq of sodium supplied is similar to the patient's original

sodium pool: $140 \times 75 \times 0.2 = 2,100$ meq. Therefore, sodium pyruvate cannot be used.

However, esters of pyruvate are stable and pH neutral. Esters of glycerol and/or dihydroxyacetone are preferred forms of amethystic agents for this invention. The ester will be hydrolyzed to pyruvic acid by non-specific esterases present in plasma, tissues and the gastrointestinal tract. Pyruvic acid will be neutralized by the body's buffers. Then, pyruvate will be reduced in the liver to lactate which will be oxidized to CO_2 in peripheral tissues. Overall, the strong pyruvic acid will be replaced by a weak acid which is eliminated by the lungs. From an acid-base point of view, this is similar to the oxidation to CO_2 of neutral compounds such as glucose and triacylglycerols. These oxidations pass through strong acids: pyruvic, lactic, acetoacetic and R- β -hydroxybutyric which are converted to weak and volatile CO_2 . Thus, as long as the rate of pyruvate ester infusion matches the capacity of peripheral tissues to oxidize lactate, the concentration of the latter could be kept at a safe level (<10 mM), without major acid-base disturbance. The transient increase in the anion gap would not be greater than what occurs after strenuous muscular exercise.

25 DPAG and PADA

A second set of the inventive compounds are dipyruvyl-acetyl-glycerol (DPAG) and pyruvyl-acetyl-

5 dihydroxyacetone (PADA). As with PNACE, these compounds are metabolizable substrates which counteract the effects of reperfusion injury. Glycerol is a physiological substrate which is well tolerated in large amounts and although DHA is not known to exist as such in body fluids, it is quickly phosphorylated by liver glycerol kinase to dihydroxyacetone phosphate (DHAP) which is a glycolytic intermediate. Similarly DPAG and PADA can be infused in vivo to deliver a therapeutic concentration of pyruvate without lactic acidosis and sodium overload. However, because DPAG and PADA can be administered in very high doses, they are also agents for accelerating ethanol oxidation in the liver, via transfer of reducing equivalents to peripheral tissues in the form of lactate.

Glycerol is a physiological substrate. It is released by adipose tissue lipolysis and is taken up by the liver, which is the major site of glycerol kinase activity (some glycerol kinase is also present in kidney). Glycerol kinase generates glycerol-phosphate which has 3 fates: glucose, glycerides/phospholipids, and lactate. DHA is converted to physiological dihydroxyacetone-phosphate (DHAP) by glycerol kinase. Then, DHAP has the same fates as glycerol. DHA is the oxidized counterpart of glycerol.

The rate of administration of pyruvate esters should be adjusted to keep lactate concentration below 10 mM. Such lactate concentrations, in the 5 to 10 mM

range, favor the competition for oxidation with other fuels such as fatty acids and glucose. One can favor lactate oxidation by infusing a small amount of dichloroacetate (DCA, final concentration 1 mM), an
5 activator of pyruvate dehydrogenase (PDH). This drug is used for the treatment of various types of lactic acidosis, in particular those resulting from congenitally low activities of PDH in peripheral tissues.

10 Because of the particular benefits of the thiol in PNACE, a dual strategy to prevent and/or treat reperfusion injury is considered advantageous. Moreover, to safely deliver large amounts of pyruvate without sodium or nitrogen load, esters of pyruvate with
15 either glycerol or dihydroxyacetone, i.e. DPAG or PADA can be used. PNACE is not a means to supply large amounts of pyruvate, since pharmacologically NAC concentrations of below 0.1 mM are often desirable, while effective pyruvate concentrations are 1- 2 mM.
20 Thus, pyruvate-glycerol or pyruvate-DHA ester is infused in large amounts together with smaller amounts of PNACE.

Acceleration of ethanol oxidation

Rat livers were infused with ethanol and the components of the esters of glycerol-and-DHA pyruvate to
25 represent the conditions that will occur after ester hydrolysis. Livers from 24 h-fasted rats were perfused with non-recirculating buffer containing 4 mM glucose

and 2 mM ethanol (20 times the K_m of ADH for ethanol, to insure zero order kinetics). After 10 min baseline, the influent perfusate was enriched with various equimolar concentrations of the components of the esters, ie DHA + Na-pyruvate, or glycerol + Na-pyruvate (up to 2.2 mM). These conditions simulated infusion and hydrolysis of glycerol- or DHA-monopyruvate. The uptakes of ethanol, pyruvate, DHA and glycerol, as well as the productions of lactate and glucose were measured.

10 Addition of the components of the pyruvate esters increased ethanol uptake up to 5 fold (Figs 3 and 4). As expected, the uptake of ethanol was greater in the presence of DHA than in the presence of glycerol. This clearly shows that DHA contributes to the trapping of
15 reducing equivalents derived from ethanol oxidation. In perfusions with glycerol + pyruvate, correlation between pyruvate uptake and lactate output was linear with a slope of 0.7 (Fig 5, solid circles). Thus, 70% of the pyruvate taken up was converted to lactate. In
20 perfusions with DHA + pyruvate (Fig 5, open circles), the correlation had also a slope of 0.7 up to a pyruvate uptake of 13 $\mu\text{mol}/\text{min}\cdot\text{g}$ dry wt (corresponding to influent DHA and pyruvate concentrations of 0.7 mM). At higher DHA and pyruvate concentrations, the slope
25 increased to 1.45. However, at the highest DHA and pyruvate concentration used, the ratio (lactate release)/(pyruvate uptake) was 0.96. The fraction of pyruvate uptake not accounted for was presumably

converted to glucose and CO_2 . Fig 6 shows that the uptake of glycerol and DHA increased with their concentration in the perfusate. As long as the (lactate production)/(pyruvate uptake) ratio was less than 1.0, there was no net conversion of glycerol or DHA to lactate. This occurred only at high DHA concentration. Thus, most of the glycerol and DHA were converted to glucose, glycerides, CO_2 or to a combination of these species. Fig. 7 shows the relationship between ethanol uptake and lactate production. Lactate yield was lower when pyruvate was infused with DHA rather than glycerol.

Before the infusion of the components of the pyruvate esters, the effluent [lactate]/[pyruvate] ratio could not be measured with precision, but must have been very high given the presence of ethanol. As the concentrations of the ester components increased from 0.4 to 2.2 mM, the [lactate]/[pyruvate] ratio went down from about 12 to about 2. Thus, essentially all reducing equivalents generated from ethanol were exported as lactate. The oxidized status of the liver NADH/NAD⁺ system may have allowed oxidation of part of the substrates, including acetate derived from ethanol.

In summary, these experiments confirmed that ethanol oxidation is stimulated by the components of DHA-pyruvate and glycerol-pyruvate. DHA is preferred as it acts not only as an esterifying group for pyruvate but also as a trap for reducing equivalents in its own right.

Therapeutic pyruvate concentration in vivo with DPAG

After preparing pure DPAG, experiments were performed to test whether it could be used to impose a therapeutic concentration of 1 mM pyruvate in arterial blood. Overnight-fasted rats, anesthetized with halothane, were infused in the jugular vein with DPAG at $90 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$ for 90 min. This rate corresponds to about 120% of the rats' caloric requirement. Five blood samples (70 μl) were taken from the carotid artery between 60 and 90 min. The arterial concentrations of pyruvate, lactate, and glycerol were clamped at 1.0, 2.5, and 0.8 mM, respectively. Corresponding portal vein concentrations at 90 min were 0.6, 2.5, and 1.0 mM, respectively. Figures 8 and 9 show blood concentrations of metabolites in rats infused with DPAG (panel A) and in control rats infused with saline (panel B). Control rats show normal arterial concentrations of pyruvate (0.05 mM) and lactate (0.3 to 0.6 mM; normal values for lactate are up to 1.5 mM). In rats infused with DPAG, the arterial concentrations of pyruvate and lactate were clamped at 1.0, and 2.5 mM, respectively. Corresponding portal vein concentrations at 90 min were 0.6 and 2.5 mM, respectively. Arterial glucose remained at 5-6 mM. Final samples of aortic blood showed normal acid-base and electrolyte parameters. Thus, DPAG can be safely used to set up the 1 mM target concentration of pyruvate expected to be beneficial for the treatment of reperfusion injury. Similar data were obtained when

PADA was infused to rats. This was achieved without sodium overload and/or acid-base perturbations. Second, the lack of major increases in glucose and lactate concentrations shows that administration of DPAG at 120% of the caloric requirement spares endogenous energy sources, probably including proteins. Third, during peripheral administration of DPAG at $90 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, portal pyruvate concentration was about 2/3 that which yielded a 3 to 6-fold increase in ethanol uptake by perfused rat livers. A portal pyruvate concentration of 1 mM could be achieved (i) by increasing the peripheral infusion of DPAG to $120 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, or (ii) by administering DPAG enterally to better target portal vein concentrations.

DPAG can thus be safely used to set up the 1 mM target concentration of arterial pyruvate expected to be beneficial for the treatment of ethanol overdose and reperfusion injury. This was achieved without sodium overload and/or acid-base perturbations. Also, the lack of major increases in glucose and lactate concentrations shows that administration of DPAG at 120% of the caloric requirement spares endogenous energy sources, probably including proteins.

The effect of DPAG on the rate of ethanol uptake by perfused rat livers was also tested. Livers were perfused with non-recirculating buffer containing 4 mM glucose, 2 mM ethanol and variable concentrations of DPAG (0 to 1.5 mM). Fig. 10 shows that the uptake of

ethanol by the liver increases 2.5 fold when DPAG concentration is raised from zero to 0.5 mM. Note that 0.5 mM DPAG corresponds to 1 mM pyruvate after hydrolysis. Thus, to accelerate ethanol oxidation in vivo, the rate of DPAG administration should be adjusted to achieve a 1 mM concentration of free pyruvate in the portal vein. When DPAG was infused to live rats at $90 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, the portal vein concentration of pyruvate was 0.6 mM. A portal pyruvate concentration of 1 mM could be achieved in vivo (i) by increasing the peripheral infusion of DPAG to $120 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}$, or (ii) by administering DPAG enterally to better target portal vein concentrations.

Accordingly, DPAG and PADA are effective in the treatment of alcoholic coma to prevent complications such as brain damage, hypothermia, respiratory depression, and pulmonary infection and in the oral intake of the esters in conjunction with ingestions of alcoholic beverages, to accelerate ethanol oxidation and restore the capacity to drive a vehicle or operate machinery.

Synthesis of DPAG

DPAG was prepared by esterification of 1-acetyl-glycerol (1-monoacetin) with pyruvyl chloride. To a 250 ml three-neck flask fitted with a thermometer, a mechanical stirrer, a 25 ml dropping funnel, and flushed with dry nitrogen, one adds 5.0 g of anhydrous

monoacetin (dried for 2 days under vacuum), .0 ml of anhydrous pyridine, and 100 ml of anhydrous benzene. The flask is cooled below 10°C with an ice + salt slurry. Freshly distilled pyruvyl chloride (6.0 ml, 1
5 equivalent) is added dropwise over 15 min, while maintaining the temperature below 10°C. Then, the reaction mixture (showing a white precipitate of pyridinium chloride) is stirred for 1 hr at room temperature. The reaction mixture is filtered, to
10 remove the pyridinium salt, and concentrated at 30°C on a rotavapor under high vacuum. The crude yellow product is dissolved in 50 ml of chloroform, washed once with 10 ml of HCl 1N, and stirred with 4g of Amberlyst-15 for 4 hr. The solvent is evaporated on a rotavapor under high
15 vacuum at 30 °C maximum. The yield of DPAG (light yellow oil) is 9.6 g (94%).

The formula of DPAG was verified by (i) NMR ¹H and ¹³C, (ii) infrared spectra, (iii) enzymatic assay of the components of DPAG after hydrolysis, and (iv) HPLC
20 before and after hydrolysis.

NMR ¹H (200 MHz Varian), solvent CDCl₃, reference TMS (δ in ppm):

5.30 (m, 1H, CH); 4.50-4.00 (m, 4H, CH₂O); 2.40 (s, 6H, CH₃COCO); 2.00 (s, 3H, CH₃CO)

25 NMR ¹H in agreement with the formula and the theoretical NMR spectra software ACD/LABS DEMO.

NMR ¹³C (200 MHz Varian), solvent CDCl₃, reference TMS (δ in ppm):

188.9, 188.7 (2C, carbonyls); 170.2 (1C, acetyl); 157.8, 157.9 (2C, pyruvyl); 68.9 (1C, CH); 61.5, 59.4 (2C, CH₂O); 24.6 (2C, CH₃); 18.4 (1C, CH₃) in agreement with the formula and the theoretical NMR spectra software
5 ACD/LABS DEMO.

IR (cm⁻¹, CCl₄) : 3537 (OH bonds from hydrated C=O), 2984; 1756, 1751, 1740, 1736, 1729 (C=O); 1383, 1231.

The NMR and IR spectra show that two molecules of water are fixed on carbonyl groups to form stable
10 hydrated keto esters.

Incubation of DPAG with pig liver esterase liberates the components of the ester which were determined by enzymatic assays, thus confirming the formula of DPAG.

15 Synthesis of PDAG

PDAG was prepared in 81% yield by reacting diacetyl-glycerol with pyruvyl chloride, using the above procedure.

The formula of PDAG was verified by (i) NMR ¹H and
20 ¹³C, (ii) infrared spectra, (iii) enzymatic assay of the components of DPAG after hydrolysis, and (iv) HPLC before and after hydrolysis.

NMR ¹H (200 MHz Varian); solvent: CDCl₃, reference TMS (δ in ppm):

25 5.28 (m, 1H, CH); 4.38-4.14 (m, 4H, CH₂O); 2.41 (s, 3H, CH₃COCO); 2.01 (s, 6H, CH₃CO). NMR ¹H in agreement with

the formula. NMR ^{13}C (200 MHz Varian), solvent: CDCl_3 , reference TMS (δ in ppm):

190.9 (1C, carbonyl); 170.4 (2C, acetyl); 159.8 (1C, pyruvyl); 71.5 (1C, CH acetyl); 68.6 (1C, CH pyruvyl);
5 61.9, 61.8 (2C, CH_2O); 24.7 (1C, CH_3 pyruvyl); 20.6 (2C, CH_3 acetyl) in agreement with the formula. IR (cm^{-1} , CCl_4) : 3593 (OH bond, from hydrated $\text{C}=\text{O}$), 2973, 1762 ($\text{C}=\text{O}$ bond), 1752 ($\text{C}=\text{O}$ bond), 1744 ($\text{C}=\text{O}$ bond), 1736 ($\text{C}=\text{O}$ bond), 1374, 1242.

10 The NMR and IR spectra show that on a small fraction of the molecules, one molecule of water is fixed on a carbonyl group to form a stable hydrated keto ester.

Synthesis of PADA

15 PADA was prepared in 95% yield by esterification of dihydroxyacetone monoacetyl with pyruvyl chloride, as above.

TLC on silica (developed with chloroform/methanol/hexane 12/1/1 and revealed with
20 iodine) showed one spot corresponding to PADA (R_f 0.45-0.50), and no dihydroxyacetone, dihydroxyacetone monoacetate, or diacetate.

The formula of PADA was verified by (i) NMR ^1H and ^{13}C , (ii) infrared spectra, (iii) enzymatic assay of the
25 components of PADA after hydrolysis, and (iv) HPLC before and after hydrolysis.

NMR ^1H (200 MHz Varian), solvent CDCl_3 , reference TMS (δ in ppm):

PADA (keto form) :4.94 (s, 2H, CH_2OCOCO); 4.74 (s, 2H, CH_2OCO); 2.49 (s, 3H, CH_3COCO); 2.08 (s, 3H, CH_3CO). NMR

5 ^{13}C (200 MHz Varian), solvent CDCl_3 , reference TMS (δ in ppm):

PADA (keto form) :198.0 (1C, keto of DHA); 192.9 (1C, keto of pyruvyl); 170.1 (1C, acetyl); 159.1 (1C, pyruvyl); 67.3, 66.3 (2C, CH_2O); 26.7 (1C, CH_3 ,

10 pyruvyl); 20.3 (1C, CH_3 acetyl). Spectra in agreement with the formula. Enzymatic assay of pyruvate after hydrolysis was in agreement with the formula.

Thus it is apparent that there has been provided, in accordance with the invention, a novel pyruvate
15 compound and a method of treating ischemia that fully satisfies the objects, aims and advantages set forth above. While the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications, and
20 variations will be apparent to those skilled in the art in light of the foregoing description. Accordingly, it is intended to raise all such alternatives, modifications and variations as fall within the spirit and broad scope of the appended claims.

WHAT IS CLAIMED:

1. A method for administering pyruvate to mammals which method comprises orally or intravenously administering a therapeutically effective amount of a pyruvate compound in the form of a pyruvate thiolester,
5 a glycerol-pyruvate ester, or a hydroxyacetone-pyruvate ester.
2. The process of claim 1, wherein said pyruvate compound is dipyruvyl-acetyl-glycerol.
- 10 3. The process of claim 1, wherein said pyruvate compound is pyruvyl-acetyl-dihydroxy acetone.
4. The process of claim 1, wherein said pyruvate compound is pyruvyl-diacetyl-glycerol.
5. The process of claim 1, wherein said pyruvate
15 thiolester is cysteine or homocysteine.
6. The process of claim 5, wherein said pyruvate thiolester is a N-acetyl derivative thereof.
7. The method of claim 1, wherein said pyruvate compound is administered as a caloric foodstuff.

8. The method of claim 1, wherein said pyruvate compound is administered as a means for lowering blood cholesterol levels.

9. The method of claim 1, wherein said pyruvate
5 compound is administered to treat an ischemic event.

10. The method of claim 1, wherein said pyruvate compound is administered to provide an energy source to an injured organ.

11. The method of claim 1, wherein said pyruvate
10 compound is administered to treat alcohol intoxication.

12. A process for preserving tissue deprived of oxygen comprising treating said tissue with a an effective amount of a solution containing a pyruvate thiolester, a glycerol-pyruvate ester, or a
15 hydroxyacetone-pyruvate ester.

13. The process of claim 12, wherein pyruvate compound is dipyruvyl-acetyl-glycerol.

14. The process of claim 12, wherein said pyruvate compound is pyruvyl-acetyl-dihydroxy acetone.

20 15. The process of claim 12, wherein said pyruvate compound is pyruvyl-diacetyl-glycerol.

16. The process of claim 11, wherein said pyruvate compound is a pyruvate thiolester of cysteine or homocysteine.

17. A method for treating ischemic tissues of a human which comprises treating said human, orally, intravenously, or intra-arterially with an effective dosage of a pyruvate thiolester compound and a glycerol-pyruvate or hydroxyacetone-pyruvate compound.

18. The method of claim 17, wherein said pyruvate thiolester includes cysteine or homocysteine.

19. The method of claim 18, wherein said pyruvate thiolester includes N-acetyl or N-acyl derivative thereof.

20. The method of claim 17, wherein said glycerol-pyruvate is dipyruvyl-acetyl-glycerol.

21. The method of claim 17, wherein said hydroxyacetone-pyruvate compound is pyruvyl-acetyl-dihydroxyacetone.

22. The method of claim 17, wherein said effective dosage comprising a therapeutic concentration between .02 and 0.1 mM thiol moiety and between 0.5 and 2.0 mM pyruvate moiety.

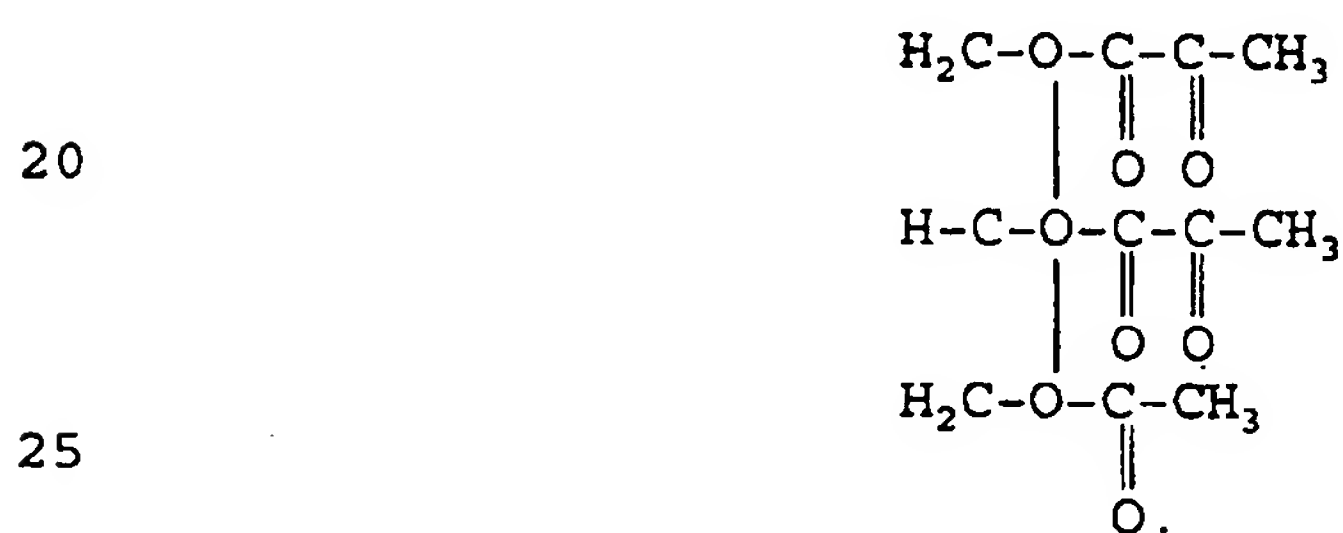
23. An organ preservation solution suitable for long term preservation of organs deprived of oxygen for transplantation, said solution including a pyruvate thiolester, a glycerol-pyruvate ester, or a hydroxyacetone-pyruvate compound.

24. The solution of claim 23, wherein said glycerol-pyruvate is of the formula



where one, two, or three R groups are pyruvyl and one or two R groups may be a short-chain acyl such as acetyl or propionyl.

25. The solution of claim 23, wherein said glycerol-pyruvate is of the formula



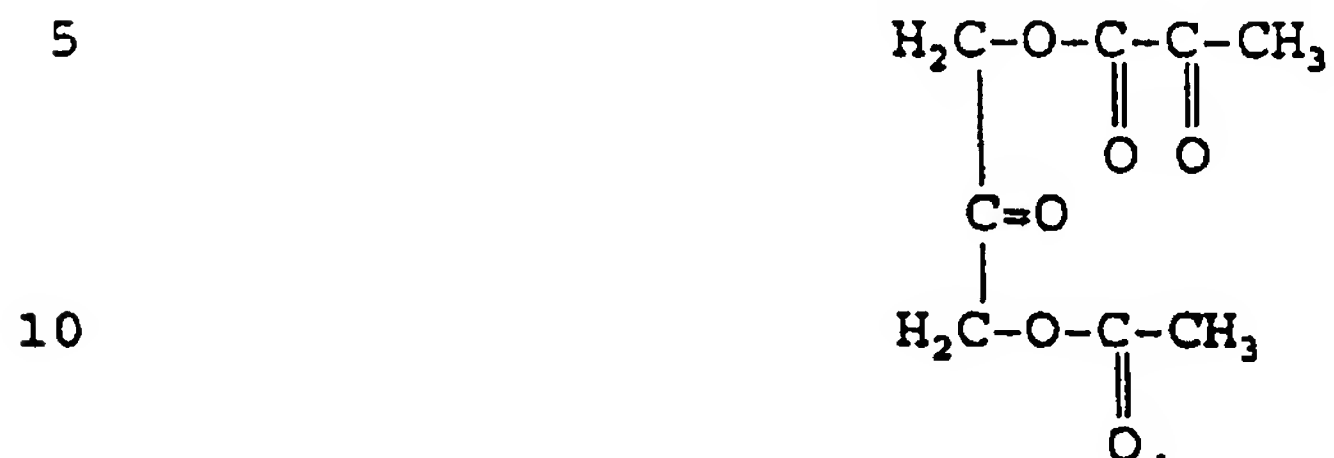
26. The solution of claim 23, wherein said hydroxyacetone-pyruvate is of the formula



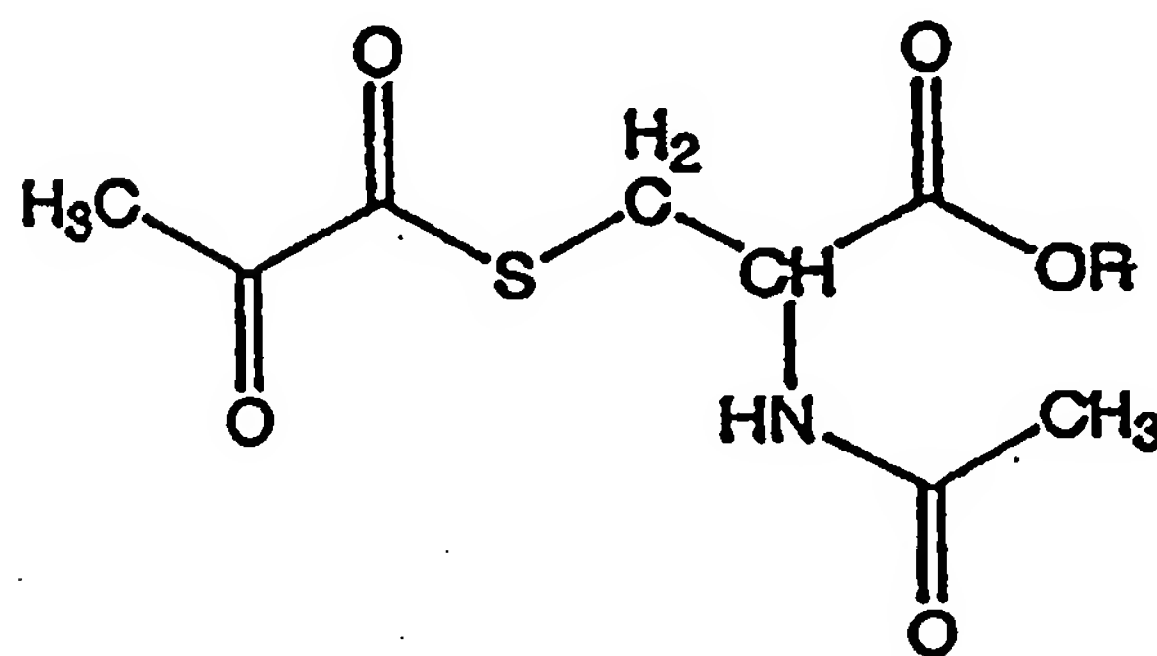
- 44 -

where one or two R groups are pyruvyl and one R group may be a short-chain acyl such as acetyl or propionyl.

27. The solution of claim 23, wherein said hydroxyacetone-pyruvate is of the formula

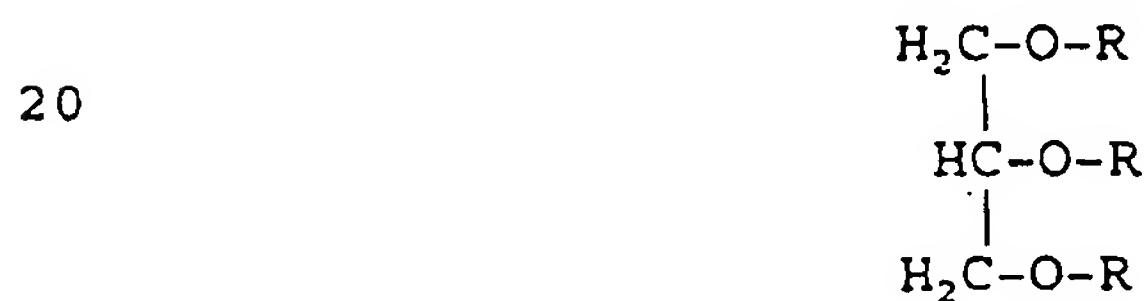


28. The solution of claim 23, wherein said pyruvate thiolester is of the formula



15 wherein R is selected from ethyl, methyl and alkyl groups.

29. A compound for administering pyruvate to humans comprising the formula



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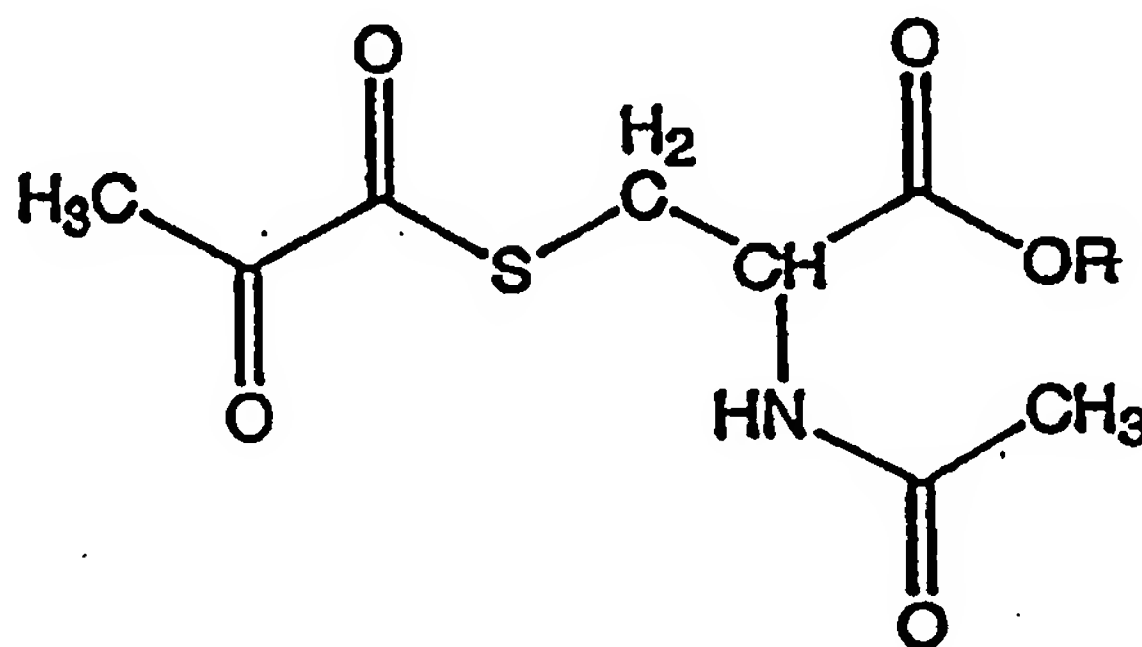
where one, two, or three R groups are pyruvyl and one or two R groups may be a short-chain acyl such as acetyl or propionyl.

30. A compound for administering pyruvate to humans comprising the formula



where one or two R groups are pyruvyl and one R group may be a short-chain acyl such as acetyl or propionyl.

31. A compound for administering pyruvate to humans comprising the formula



wherein R is selected from ethyl, methyl and alkyl groups.

32. A method of treating acetaminophen poisoning according to claim 1.

Cardiac Recovery after 25 min. Global Warm Ischemia

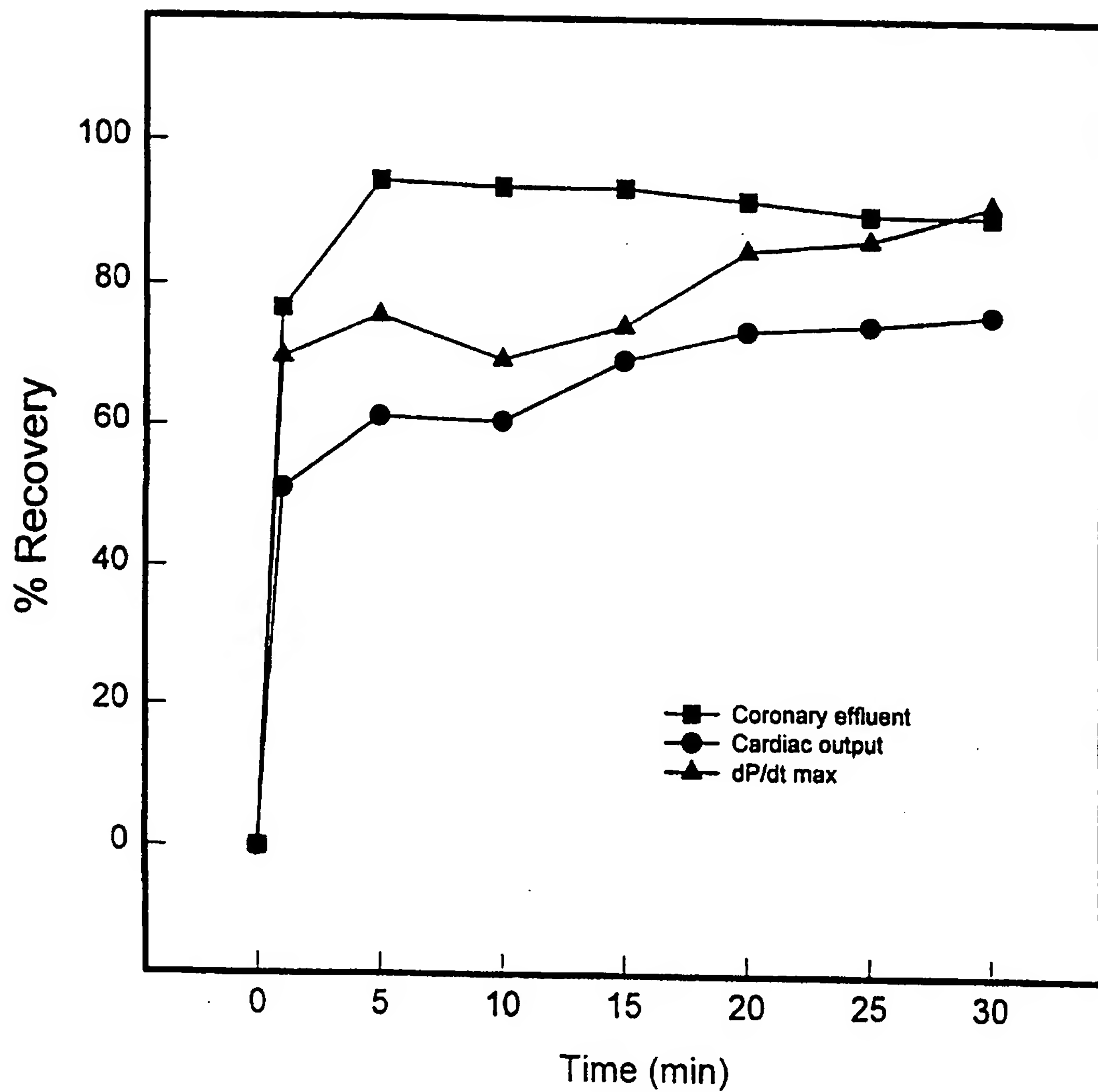


Fig. 1

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Cardiac Recovery after 25 min. Global Warm Ischemia

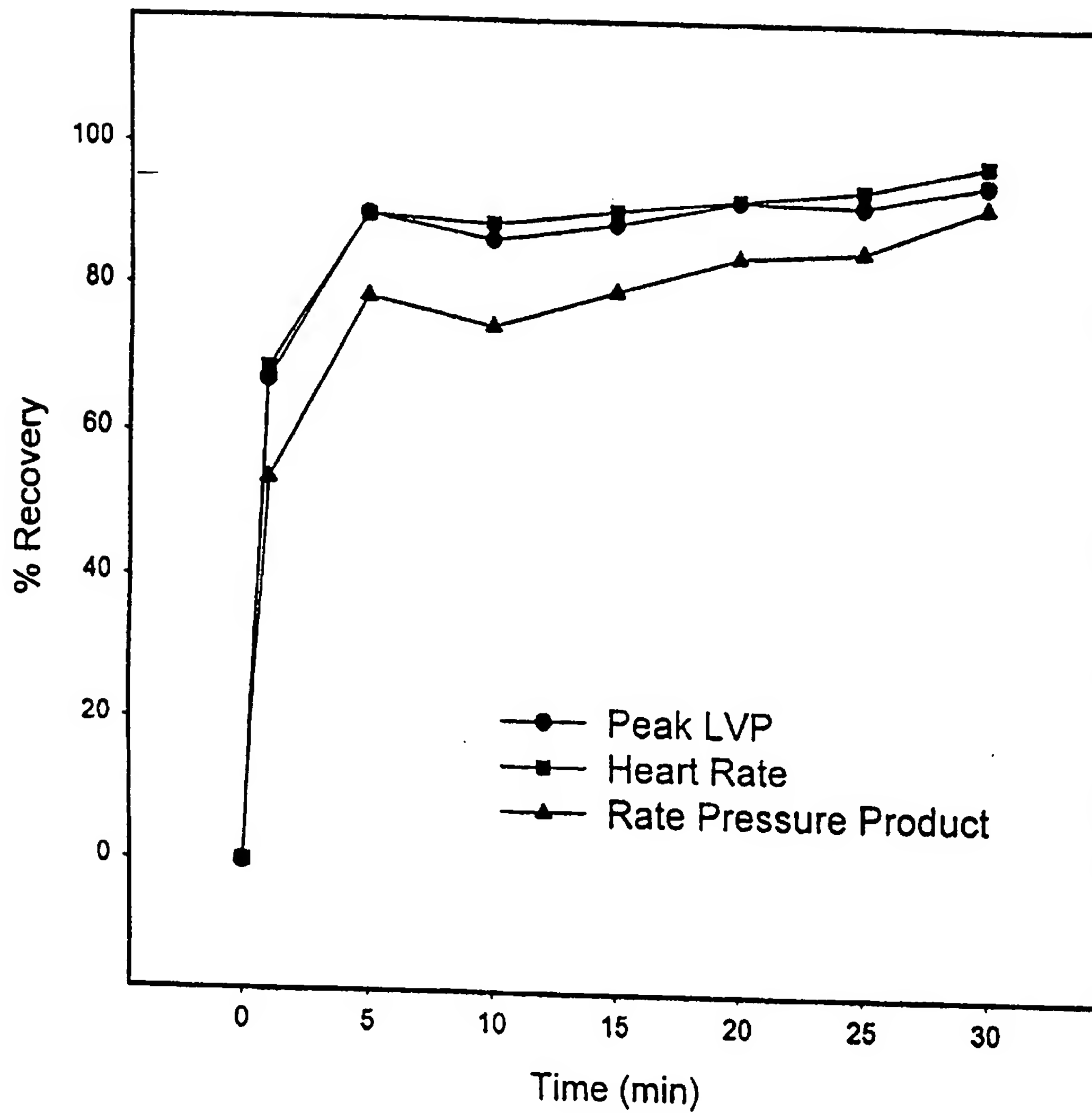


Fig. 2

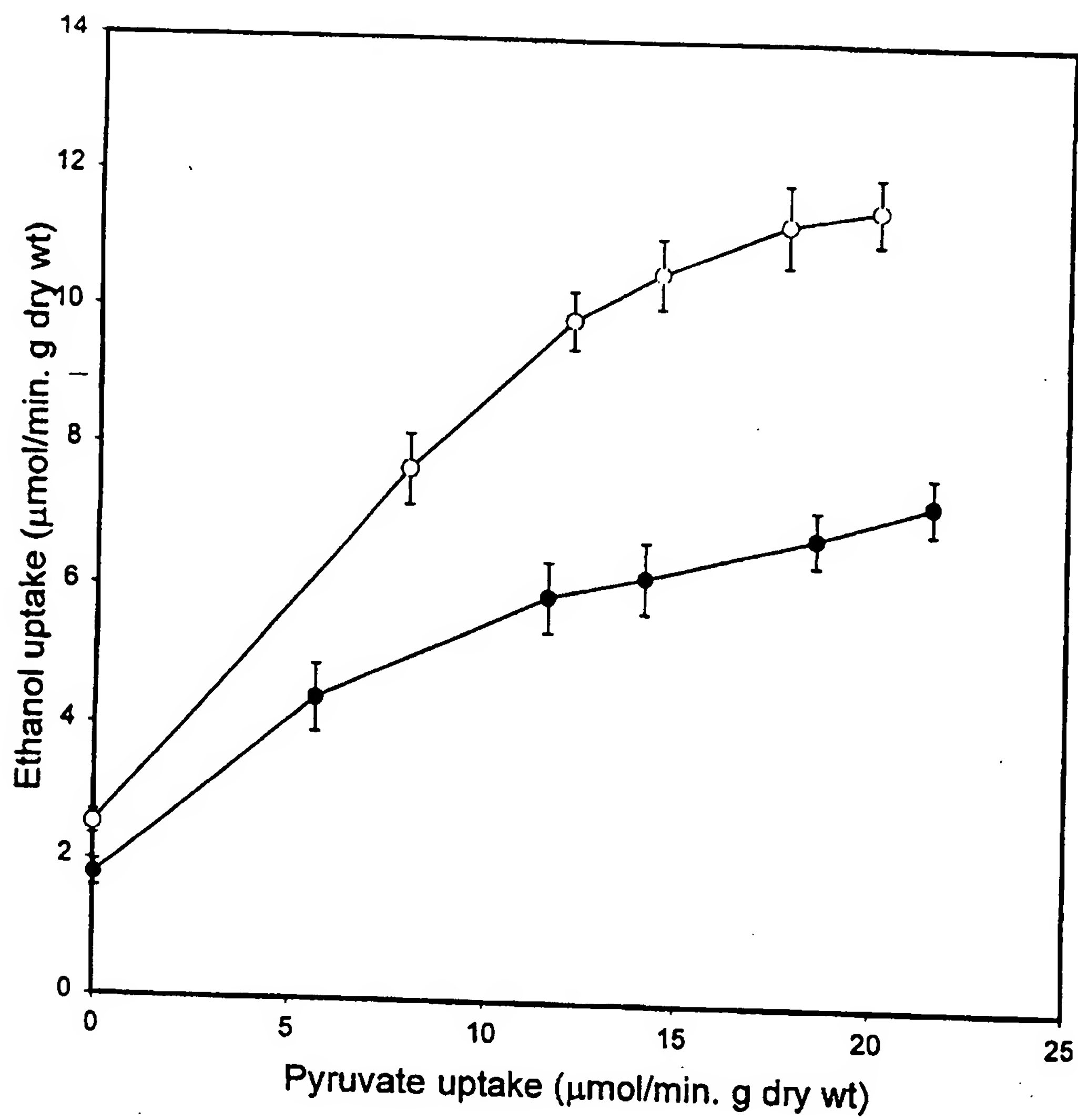


Fig.3

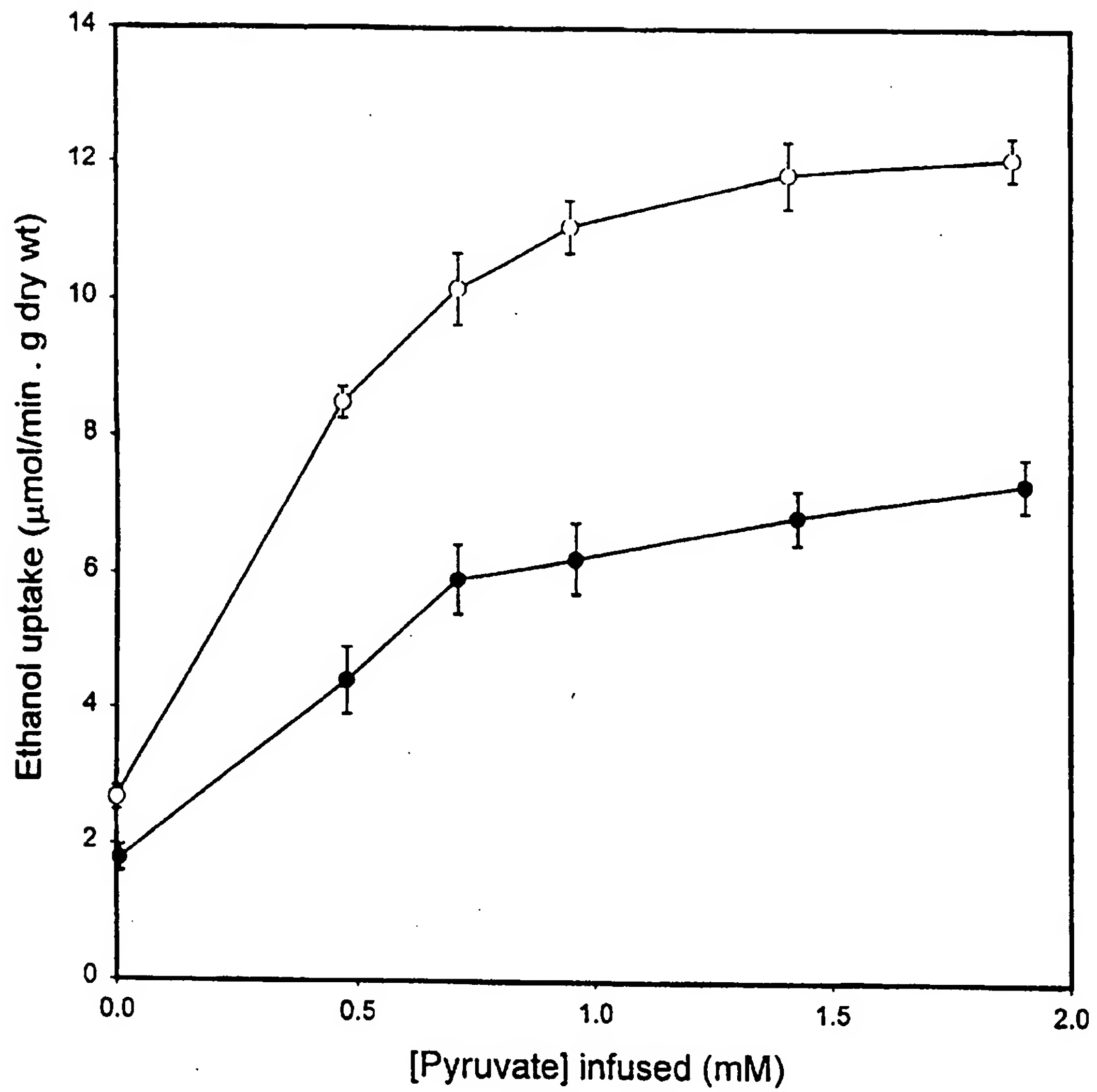


Fig. 4

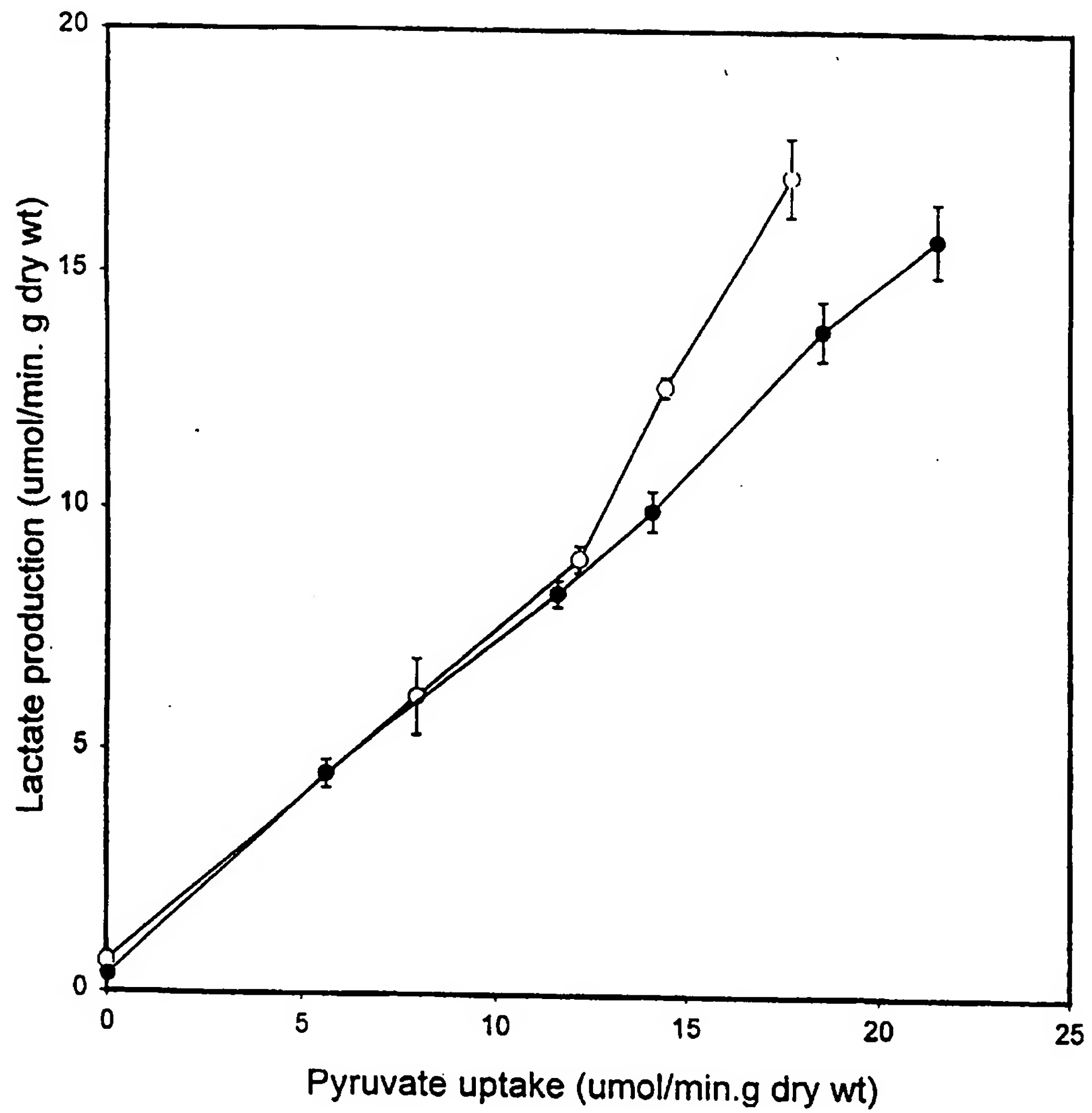


Fig. 5

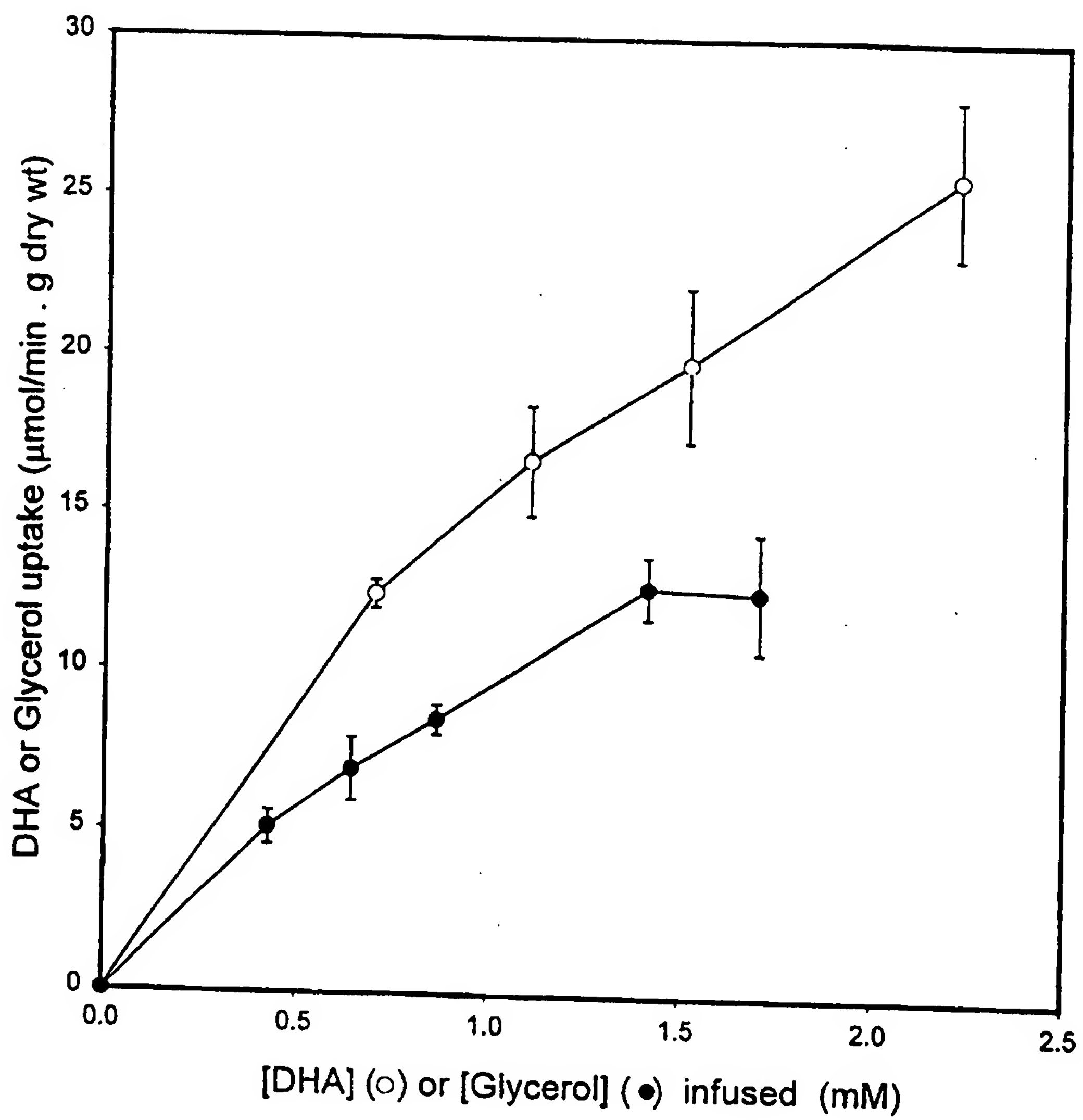


Fig. 6

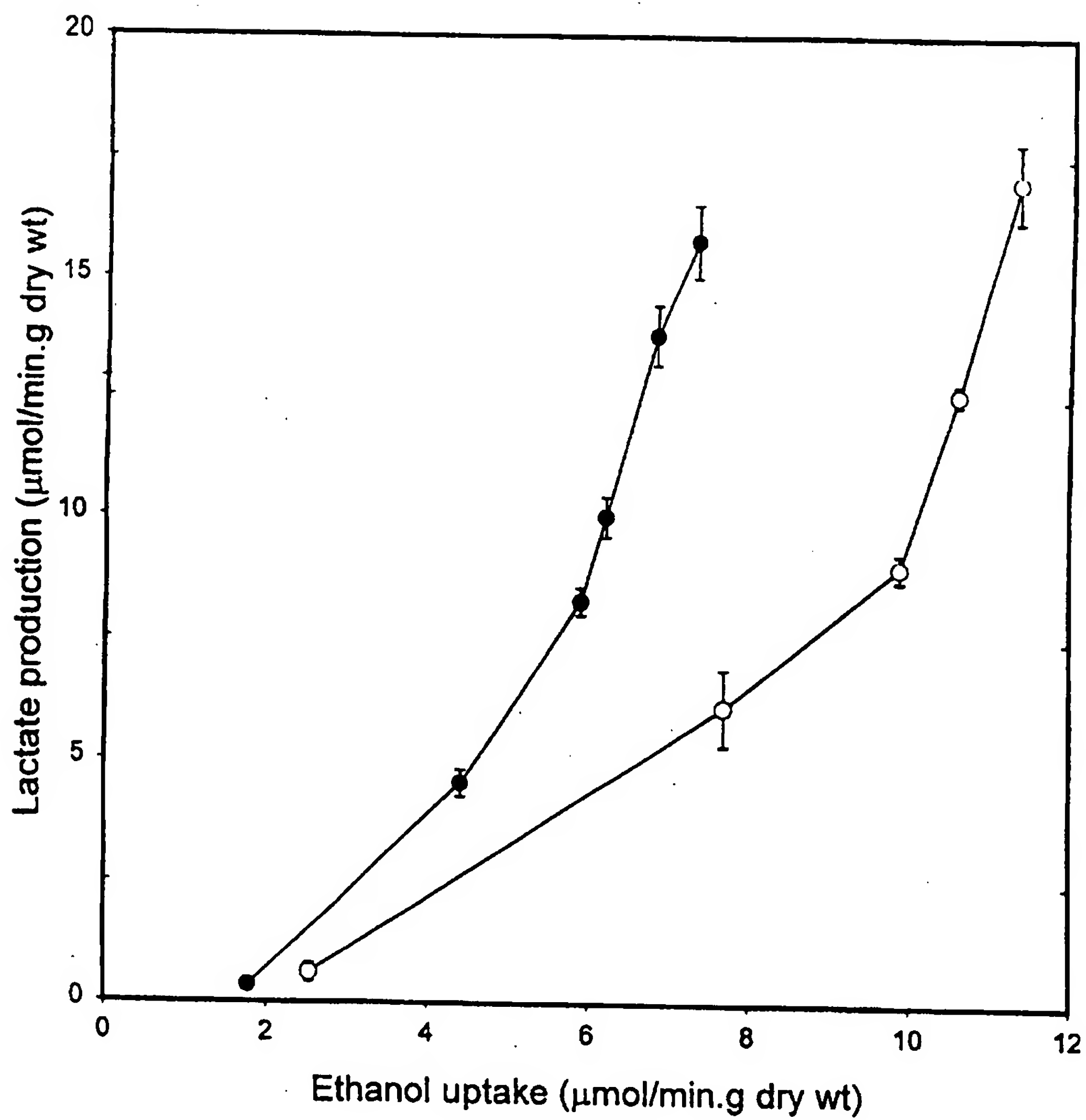


Fig. 7

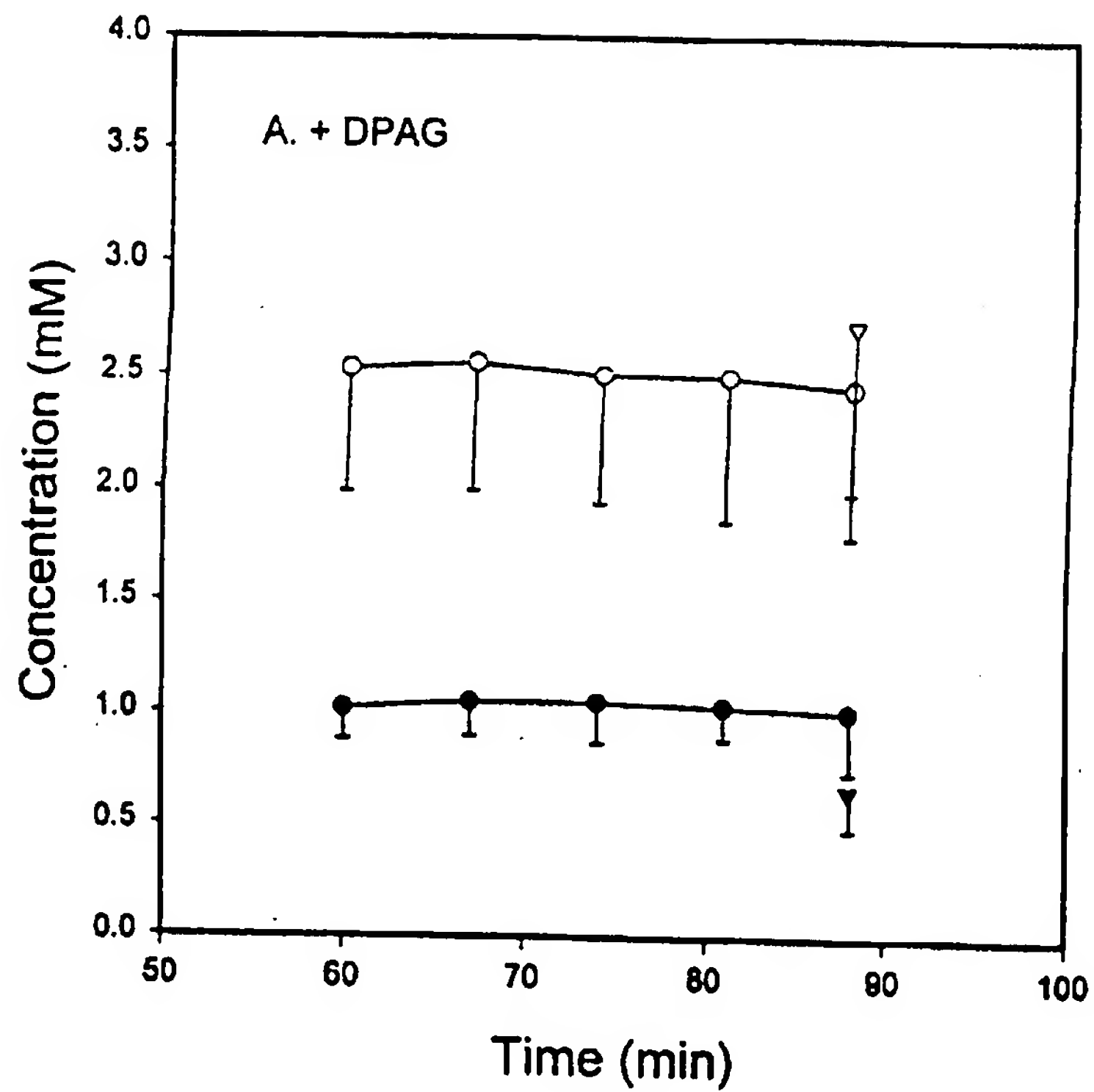


Fig. 8

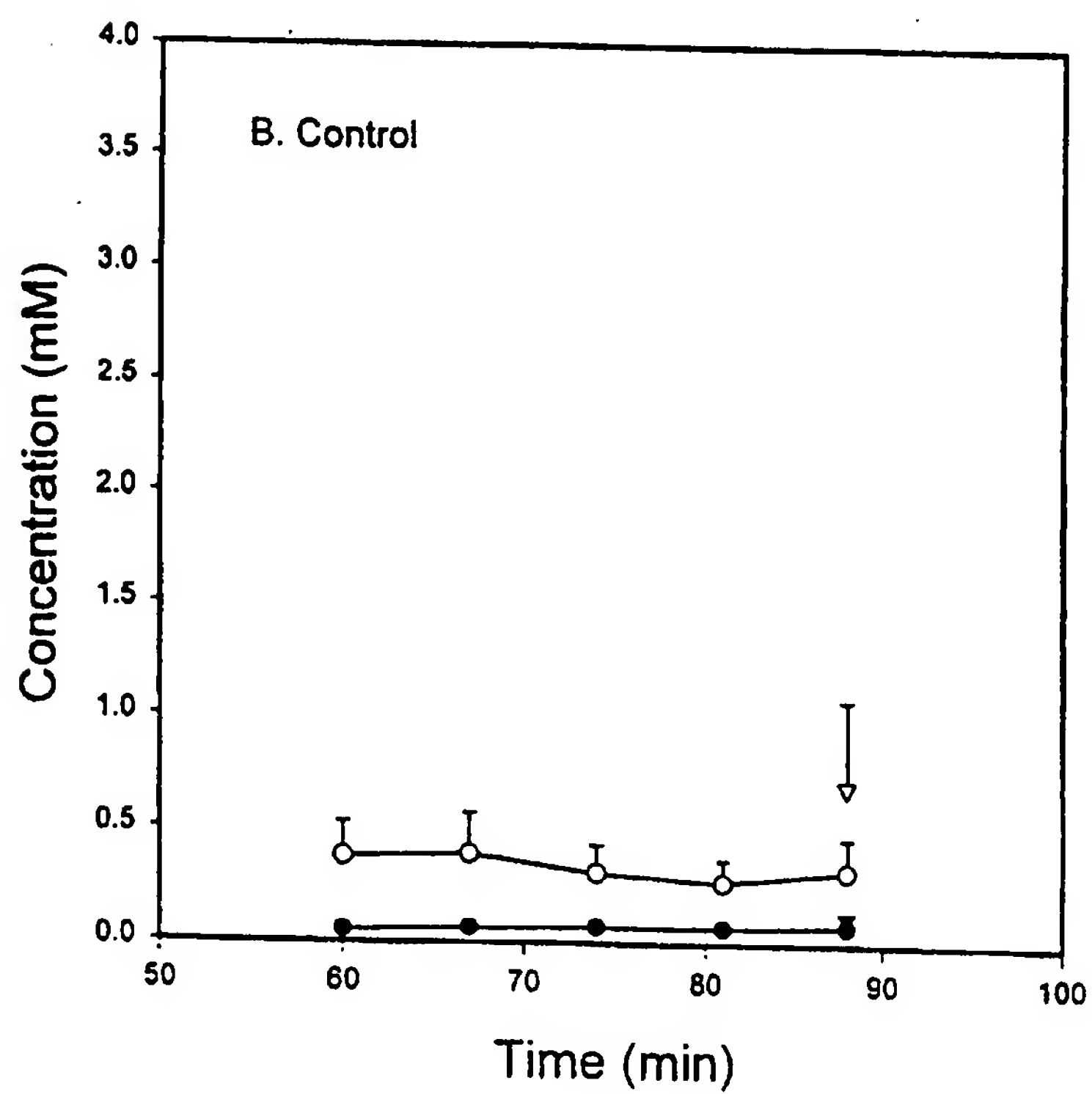


Fig. 9

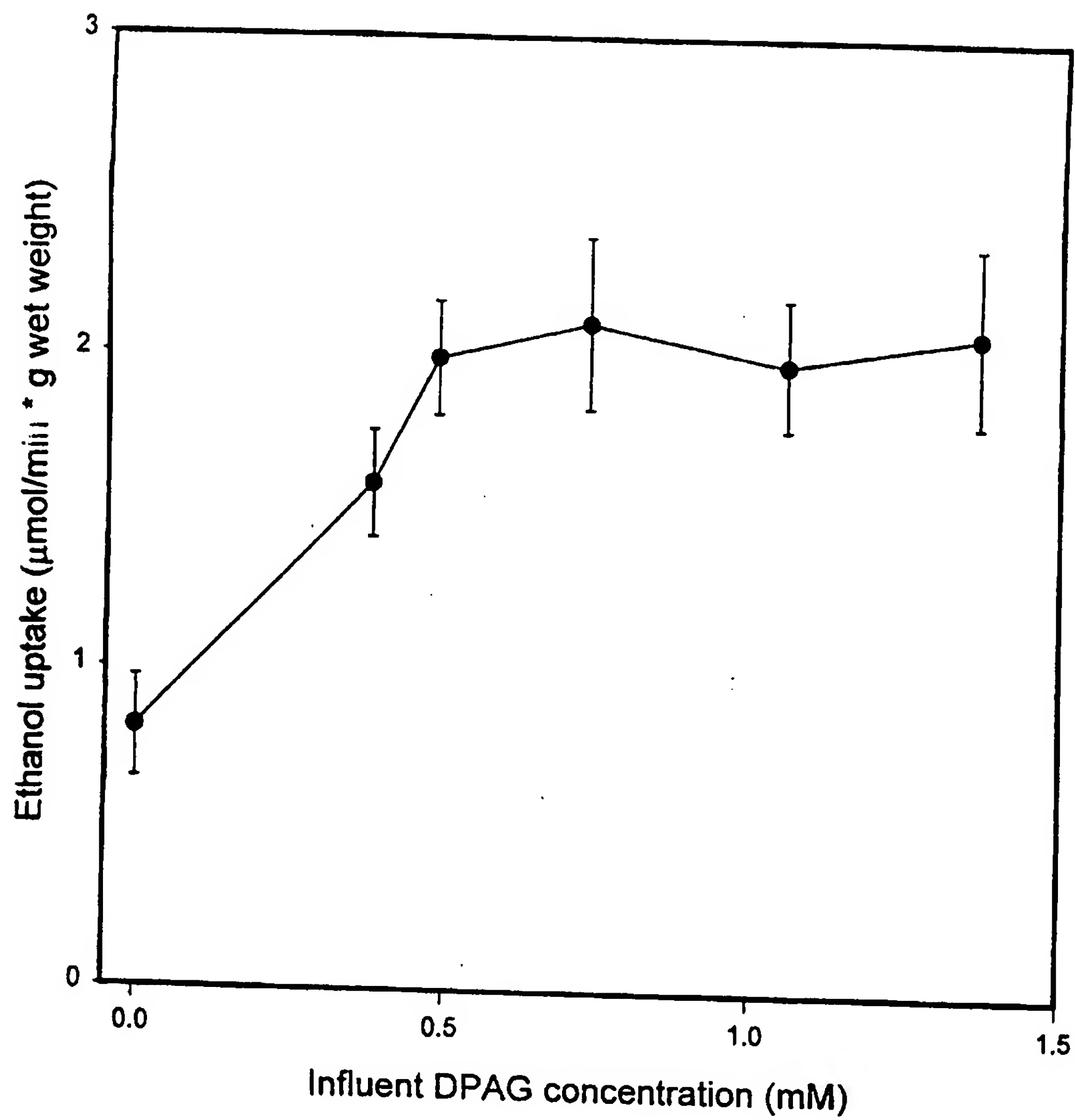


Fig. 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04335

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07C 31/22, 59/245, 59/285, 69/66, 229/30; A01N 1/02

US CL : 435/1; 514/506, 529, 546; 554/115, 227; 560/155, 174

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/1; 514/506, 529, 546; 554/115, 227; 560/155, 174

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE

search terms: pyruv?, thiolester, glycer?, heart, organ, perfusion

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,283,260 A (MILLER ET AL.) 01 February 1994 (01.02.94), page 1, abstract.	1-11 and 16-21
X, Y	US 5,294,641 A (STANKO) 15 March 1994 (15.03.94), column 3, lines 24-47.	1-11 and 16-32
Y	US 5,075,210 A (WIKMAN-COFFELT) 24 December 1991 (24.12.91), page 1, abstract.	12-15

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

•	Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A	document defining the general state of the art which is not considered to be of particular relevance		
*E	earlier document published on or after the international filing date	*X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O	document referring to an oral disclosure, use, exhibition or other means		
*P	document published prior to the international filing date but later than the priority date claimed	*&	document member of the same patent family

Date of the actual completion of the international search

09 MAY 1997

Date of mailing of the international search report

27 JUN 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Authorized officer

JANE OSWECKI

Telephone No. (703) 308-1235

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04335

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☐

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/04335

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

Group I, claims 2-4, 12-15, 20, 21, 23-27, 29 and 30, drawn to optionally substituted glycerol pyruvate ester derivative compounds and optionally substituted hydroxyacetone pyruvate ester derivative compounds.

Group II, claims 5, 6, 12, 16-19, 22, 23, 28 and 31, drawn to optionally substituted pyruvate thiolester compounds.

Claims 1, 7, 8-11 and 32 are generic to any group paid for.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical feature which makes a contribution over the prior art is C(OR)-C-C(OR) in Group I and a thiolester in Group II. Each of these compounds is different, one from the other, in structure, and the compounds are not known as equivalents in the art.